MECHANISMS OF AUTOPHAGY IN METHYLOTROPHIC YEASTS

By

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KEY TO ABBREVIATIONS

AOX: alcohol oxidase
BSA: bovine serum albumin
CCO: cytochrome *c* oxidase
DHAS: dihydroxyacetone synthase
DSM: diploid selection medium
EA: ethanol adaptation medium
F1β: mitochondrial F_1 ATPase, β subunit
FBP: fructose-1,6- bisphosphatase
FDH: formate dehydrogenase
GA: glucose adaptation medium
gsa: glucose-induced selective autophagy mutant
LSM: low sulfate medium
MIM: methanol induction medium
SA: heat-killed, glutaraldehyde-fixed *Staphylococcus aureus* cells
SM: sporulation medium
TX100: triton X-100
YPD: glucose-containing complete medium

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MECHANISMS OF AUTOPHAGY IN METHYLOTROPHIC YEASTS

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Major Department: Anatomy and Cell Biology

When the yeasts *Hansenula polymorpha* and *Pichia pastoris* utilize methanol as sole carbon and energy source, peroxisomes and cytosolic enzymes which metabolize it are induced. If the cells are subsequently switched to media containing glucose or ethanol, the superfluous peroxisomes and cytosolic methanol assimilation enzymes are degraded. The purpose of the present project was to examine the process of turnover of cellular components utilizing biochemical, ultrastructural, and genetic means, thereby advancing the understanding of the mechanisms of autophagy.

Cellular proteins were radioactively labeled to show that glucose mediates the selective, enhanced degradation of methanol-induced peroxisomes. Utilizing mutant strains of *P. pastoris*, which contain null alleles for vacuolar proteinase A and proteinase B, rendering the vacuole proteolytically

inactive, it was determined that the vacuole is the site of degradation of peroxisomes and cytosolic formate dehydrogenase. Morphological examination revealed species- and nutrient-specific modes of delivery of cytoplasmic components to the vacuole for degradation. Macroautophagic sequestration of peroxisomes is induced in *H. polymorpha* by glucose and in *P. pastoris* by ethanol. In contrast, glucose initiates selective microautophagy in *P. pastoris*. Nitrogen deprivation also initiates microautophagy in *P. pastoris*, but of a morphologically distinct type which degrades cytoplasmic components nonselectively. Microautophagy in *P. pastoris* requires protein synthesis whereas macroautophagy does not. Models are presented which illustrate hypothesized steps in the ultrastructure and mechanisms of the various modes of autophagy.

In order to evaluate the molecular basis of autophagy, mutants defective in glucose-induced selective autophagy (*gsa*) were isolated and characterized. Members of two complementation groups, WDY1 and WDY2, were determined to have proteolytically active vacuoles but were unable to conduct microautophagy in response to glucose or nitrogen starvation. Macroautophagy proceeds normally in these mutant strains in response to ethanol. A genomic library clone has been isolated which functionally complements the genetic defect in microautophagy in WDY2.

This analysis of *P. pastoris* provides the first models for microautophagy in yeast. The models and tools for genetic analysis of autophagy presented

herein will be useful for evaluating the molecular mechanisms of selective and nonselective autophagy in yeast.

CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

Methanol Metabolism and Peroxisomes in Yeast

Certain species of yeast within the genera *Candida*, *Hansenula*, and *Pichia* are able to utilize methanol as the sole source of carbon and energy so are referred to as methylotrophic (Fukui et al., 1975; van Dijken et al., 1975). Many of the enzymes necessary to assimilate methanol are not present in detectable amounts in cells grown on other carbon and energy sources and consequently must be induced during adaptation to methanol-limited media. Hydrogen peroxide is one of the products of the first enzyme-mediated reaction of methanol metabolism (Figure 1-1); thus, as with other enzymes which generate hydrogen peroxide, this enzyme (alcohol oxidase; AOX) and certain others of this pathway are located within peroxisomes. Alcohol oxidase in its active form is a homo-octameric flavoprotein containing FAD as a prosthetic group (van der Klei et al., 1990). AOX has a low affinity for its substrate thus large quantities of it are necessary for the yeast to subsist on methanol (van Dijken et al., 1975; 1982; Harder et al., 1987; Gleeson and Sudberry, 1988). Crystalline inclusions composed of alcohol oxidase (AOX) form in methanol-induced peroxisomes, occupying most of the lumen of peroxisomes, which have a diameter of between 0.9 and 1.3 μm. These peroxisomes are surrounded by a

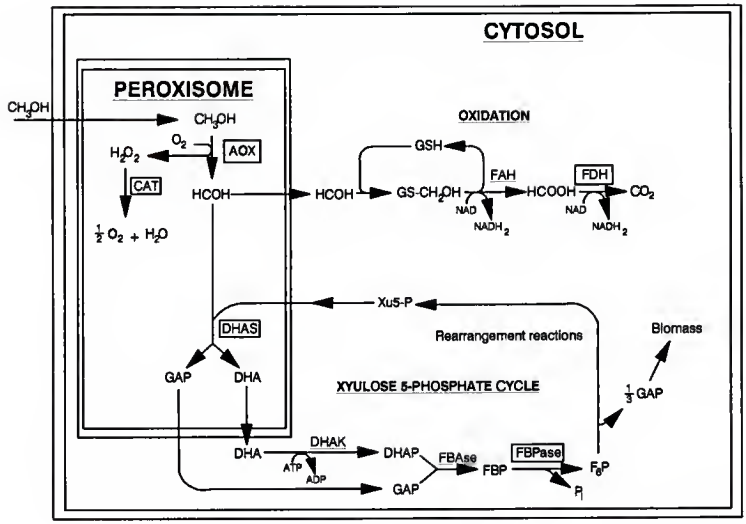

Figure 1-1. Methanol metabolism and its compartmentalization in methylotrophic yeasts. Methanol is oxidized in the peroxisome by alcohol oxidase (AOX) yielding formaldehyde and H_2O_2 which is rapidly detoxified by catalase (CAT). Formaldehyde is then either oxidized in the cytosol following interaction with reduced glutathione (GSH) by formaldehyde dehydrogenase (FAH) and formate dehydrogenase (FDH) or assimilated via the xyulose 5-phosphate cycle. The enzymes active in the xyulose 5-phosphate cycle include dihydroxyacetone synthase (DHAS) which catalyzes the reaction of xyulose 5-phosphate (Xu5-P) with formaldehyde, yielding glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone (DHA), dihydroxyacetone kinase (DHAK) yielding dihydroxyacetone phosphate (DHAP), fructose 1,6-bisphosphate aldolase (FBAse) yielding fructose bisphosphate (FBP), and fructose 1,6-bisphosphatase (FBPase) yielding fructose 6-phosphate (F_6P). By a series of rearrangement reactions, xyulose 5-phosphate is regenerated to complete the cycle. The pathway is responsible for the generation of GAP made available for production of biomass. Underlined and boxed acronyms are enzymes, boxed acronyms are enzymes which have been used as markers for degradation in the present study. Adapted from Gleeson and Sudberry (1988).

unit membrane and appear in clusters in the cell, occupying as much as 80% of cell volume under optimal growth conditions (Gleeson and Sudberry, 1988). The two peroxisomal proteins AOX and dihydroxyacetone synthase together may account for > 50% of the protein mass in a fully methanol-induced cell (see Fig. 3-1). The peroxisomes are visible even at the light level and clustered peroxisomes are the dominant feature in cells viewed by electron microscopy.

In methylotrophic yeasts, methanol is utilized in two ways: a catabolic pathway to produce energy in the form of $NADH_2$ or an anabolic pathway to produce substrates for the synthesis of biomass (Fig. 1-1; van Dijken et al., 1975;1982; Harder et al., 1987; Gleeson and Sudberry, 1988). The first step of methanol utilization takes place in the peroxisome and entails its oxidation by AOX (also referred to as methanol oxidase) the products of which reaction are hydrogen peroxide and formaldehyde. Catalase is diffused in the peroxisomal matrix and detoxifies the hydrogen peroxide formed, producing water and molecular oxygen. Formaldehyde is the substrate which can be directed down either the catabolic or anabolic pathways. Catabolism involves a linear pathway involving the complete oxidation of the formaldehyde after it diffuses into the cytoplasm. In the cytosol, formaldehyde reacts with reduced glutathione and is subsequently oxidized by formaldehyde dehydrogenase and formate dehydrogenase (Fig. 1-1, top half). NAD is reduced to $NADH_2$ in the last two steps, yielding two reducing equivalents per molecule of methanol.

Alternatively, methanol can be assimilated into cellular mass via the

xyulose 5-phosphate cycle (Fig. 1-1, bottom half). The first step in this cycle is the reaction between formaldehyde and xyulose 5-phosphate, catalyzed by the peroxisomal enzyme dihydroxyacetone synthase (DHAS). The products of this reaction, glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone, diffuse into the cytosol and undergo a series of rearrangement reactions which serve to regenerate xyulose 5-phosphate and produce 1/3 molecule of GAP available for biomass production per molecule of methanol assimilated. The methanol utilization enzymes AOX, DHAS, and formate dehydrogenase (FDH) are undetectable in cells grown on carbon sources other than methanol, e.g., glucose and ethanol.

The Yeast Vacuole

The vacuole serves as the main degradative organelle in *Saccharomyces cerevisiae*, the yeast that has been most extensively studied relative to degradation and vacuolar function. In some ways the vacuole is the functional equivalent of the mammalian lysosome: 1) Vacuolar component proteins are synthesized and directed to the vacuole via the secretory pathway and proteolytically activated in the vacuolar lumen. 2) It is acidic and contains a variety of hydrolytic enzymes. 3) It is the site of degradation of endocytosed materials. 4) Recent evidence suggests that it is also the site of degradation of intracellular components, i.e., the endpoint for materials sequestered by autophagy. In other ways it is quite different: 1) while in mammals there are

many small lysosomes in each cell, the yeast vacuole is quite large, occupying 10-20% of the cell volume, and usually there is only one, lobulated vacuole per cell; and 2) the yeast vacuole actively and precisely regulates cytosolic concentration of many different constituents.

Vacuolar Biogenesis

A discussion of the biogenesis of the yeast vacuole includes biosynthesis, sorting, targeting, and processing of the hydrolases which reside in the vacuole (Fig. 1-2; for reviews see Klionsky et al., 1990; Raymond et al., 1992). The hydrolase which has been most highly studied and has served as a model of a typical soluble vacuolar protein is carboxypeptidase Y (CPY).

Processing and sorting of vacuolar protease. Temperature-sensitive mutants in the secretory pathway, *sec61*and *sec62*, block endoplasmic reticulum (ER) translocation at the restrictive temperature, causing the accumulation of the unglycosylated, signal sequence-containing form of CPY (preproCPY). At the permissive temperature, the 20 amino acid signal peptide is proteolytically removed at the time of translocation into the ER. These data suggest that, as is usual for proteins in the secretory pathway, CPY is synthesized as a precursor that translocates into the ER at which time the N-terminal signal sequence is cleaved yielding the zymogen proCPY (Blachly-Dyson and Stevens, 1987; Johnson et al., 1987).

Dolichol-mediated glycosylation occurs in the ER with the addition of core oligosaccharides at four sites on proCPY (Trimble et al., 1983). ProCPY is

further modified in the Golgi apparatus with the elongation of the core oligosaccharides with additional mannose residues yielding the fully glycosylated Golgi precursor form (see Fig 1-2). Unlike the long mannose outer chains of secreted yeast proteins, CPY and the other vacuolar proteins undergo limited elongation. There is strong evidence that the type of glycosylation modifications is not responsible for sorting of secretory proteins away from vacuolar proteins. This evidence includes the ability of cells to correctly sort vacuolar proteins in the presence of tunicamycin, which blocks the addition of N-linked oligosaccharides to proteins (Klionsky et al., 1988). Furthermore, hybrid proteins which contain sections of vacuolar proteins fused to the secretory protein invertase are efficiently delivered to the vacuole despite the fact that the same carbohydrate elongation takes place as in the secreted wild type invertase (Johnson et al., 1987; Klionsky et al, 1988). These data suggest that the sorting signals reside in the polypeptide chains themselves. It was determined, using fusion proteins and site-directed mutagenesis, that the vacuolar targeting signal of CPY resides in the N-terminal region of proCPY (Valls et al., 1987). This is in contrast to the case of mammalian lysosomal proteins, which are diverted from the secretory path by the interaction of mannose 6-phosphate residues attached to the proteins and mannose 6-phosphate receptors in the Golgi (reviewed in Griffiths et al., 1988; Kornfeld and Mellman, 1989).

Transport of vacuolar proteases to the vacuole. Data have recently been presented which suggests that Golgi-modified vacuolar proteins do not traffic

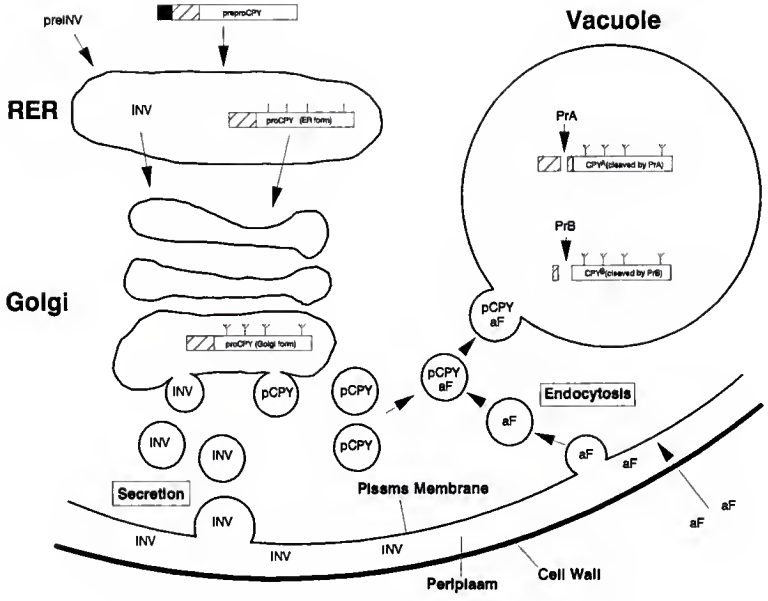

Figure 1-2. Processing, sorting, and activation of carboxypeptidase Y, a representative vacuolar protease. Newly synthesized carboxypeptidase Y (preproCPY) enters the secretory pathway after translation on the rough endoplasmic reticulum (RER) at which time the signal sequence (pre: denoted by the black box) is cleaved. N-linked core glycosylation occurs at four sites on the protein in the RER (vertical lines) which is further processed in the Golgi by addition of mannose residues (branching diagonal lines). In the late Golgi vacuolar proteins are sorted from proteins destined to be secreted (e.g., invertase = INV). Fully glycosylated CPY (proCPY or pCPY) leaves the Golgi, presumably in vesicles, which fuse with endocytic vesicles, in this case containing endocytosed alpha-factor (aF), before finally fusing with the vacuolar membrane where the pCPY is deposited into the vacuolar lumen. This inactive, zymogen form of CPY is proteolytically cleaved, in turn, by the endoproteases proteinase A (PrA) yielding active CPY^A and proteinase B (PrB) yielding the protein of mature size (CPY^B).

directly to the vacuole but rather by way of an endosome-like intermediate (Vida et al., 1993). In these studies, intercompartmental transport was reconstituted in permeabilized spheroplasts using pulse/chase protocols and subcellular fractionation. After a 5 minute pulse and 30 second chase, 30 to 40% of the Golgi-modified precursor form of CPY fractionated at a density intermediate between the vacuole and Golgi. Mutant CPY lacking a functional sorting signal was detected in Golgi fractions but not the intermediate density compartment, indicating that the intermediate density compartment is downstream of the Golgi. In addition, the Golgi-modified CPY co-fractionated with ^{35}S-α-factor internalized at 15°C. The authors concluded that CPY is sorted away from secretory proteins in the late Golgi and proceeds to the vacuole via an endosome-like intermediate (see Fig. 1-2). Davis et al. (1993) provided further evidence for convergence of the endocytic and vacuolar biogenesis pathways based on the fact that REN1, a gene which blocks α-factor receptor delivery to the vacuole, and VPS2, a gene required to deliver newly synthesized vacuolar proteases to the vacuole are identical genes.

Vacuolar biogenesis mutants. A large set of mutants has been isolated which are defective in vacuolar biogenesis (see Rothman et al., 1989 for a description of the genes involved). At least 49 genes in the PEP, END, VPL, VPS, and VPT complementation groups are required to target and deliver soluble enzymes, including CPY, to the vacuole. These genes include a member of the ras-like GTP binding family with striking similarity to mammalian

rab5 (VPS21; Horazdovasky et al., 1994); a phosphatidylinositol 3-kinase and a protein kinase acting as part of a hetero-oligomeric protein complex (VPS34 and VPS15, respectively; Stack et al., 1993); and a peripheral vacuolar membrane protein (PEP3: Preston et al., 1991; a.k.a. VPS18: Robinson et al., 1991) among others. Of these genes one of the best characterized is PEP4, encoding the vacuolar endoprotease proteinase A (PrA), which is required for the processing of vacuolar zymogens and overall proteolytic activity of the vacuole (Jones, 1984; Ammerer et al., 1986; Hirsch et al., 1992).

Processing of vacuolar zymogens. A total of seven vacuolar proteases are known: two endoproteases, PrA and proteinase B (PrB); two carboxypeptidases, CPY and carboxypeptidase S; two aminopeptidases, aminopeptidase I (AP-I) and aminopeptidase Co; and dipeptidyl aminopeptidase B (reviewed in Jones, 1991a; Knop et al., 1993). Of these PrA, PrB, AP-I, and CPY are transported to the vacuole in inactive, zymogen form while the others are transported in their active forms. The pathway of activation of these zymogens has been worked out in detail by genetic and biochemical means revealing that PrA autocatalyzes its own proteolytic cleavage to active form and then acts on PrB, AP-I, and CPY to activate them (Ammerer et al., 1986; Teichert et al., 1987; Hirsch et al., 1992). While the PrA-catalyzed cleavage will activate these proteases, this cleavage alone is not enough to process the zymogens to mature size and form; this requires a further clip by PrB, though the functional significance of this final trimming is not known (see Fig. 1-2).

The Vacuole Regulates Cytosolic Concentrations of Substrates

The vacuolar H^+ ATPase is the enzyme most responsible for generating an electrochemical potential difference of protons across the vacuolar membrane, maintaining the vacuolar lumen at ~ pH 6.0 (Preston et al., 1989). The proton gradient is the primary force for the transport of amino acids, Ca^{2+}, P_i, Zn^{2+}, Mg^{2+}, and other ions via proton antiporters (Ohsumi et al., 1981; 1983). Eight different amino acid/proton antiporters have been identified in the vacuole of *S. cerevisiae*, specific for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and leucine-isoleucine (Sato et al., 1984). While amino acid concentrations in the cytosol remain relatively constant, those in the vacuole fluctuate widely depending on availability of nutrients and stage of growth (Kitamoto et al., 1988). Arginine is usually the major amino acid stored in the vacuole, probably since it is the most nitrogen-rich amino acid and therefore serves as a nitrogen reserve. In fact, nitrogen starvation evokes a transfer of much of the vacuolar arginine pool into the cytosol.

Protein Degradation in Yeast

Proteolysis is an integral part of post-translational control that functions either by limited clips that regulate the function of a protein or total degradation to regulate the quantity of a protein or to dispose of defective proteins (see Teichert et al., 1987; Jones, 1991a; Rendueles and Wolf, 1988; Holzer, 1976).

Proteolytic activities have been found in nearly all compartments of yeast, including mitochondria, endoplasmic reticulum, cytoplasm, vacuole, and periplasm. Particular instances of limited proteolysis which activate vacuolar zymogens have been discussed above so here I will review what is known about the total degradation pathways which occur in the endoplasmic reticulum, cytoplasm, and especially the vacuole.

Protein Degradation Within the Endoplasmic Reticulum

In order to show degradation within the endoplasmic reticulum, it must be shown that lysosomal/vacuolar proteases are not involved and that the protein does not exit the endoplasmic reticulum. Protein degradation within the endoplasmic reticulum appears to include elimination of misfolded mutant proteins as well as regulation of levels of normal proteins. The control of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) levels in the endoplasmic reticulum has been studied in mammalian cells to reveal a highly regulated system modulating synthesis and degradation depending on cellular needs (Nakanishi et al., 1988). The half-life of HMGR in cultured cells varies between 40 minutes and >10 hours depending on the rate of synthesis in the mevalonate pathway of isoprenoid synthesis. Recent work in *S. cerevisiae* has suggested that HMGR levels are regulated in a manner similar to that observed in mammals and that it is normally degraded in the endoplasmic reticulum (Hampton and Rine, 1994).

The degradation of abnormal proteins in the endoplasmic reticulum has also been studied in *S. cerevisiae* (Finger et al., 1993). In this study, the fate of mutant forms of the vacuolar enzymes CPY and PrA was determined. The mutant PrA form exhibited no proteolytic activity *in vitro* while the mutant CPY was susceptible to trypsin cleavage *in vitro*, unlike the wild type form, indicating an altered structure. No Golgi-specific carbohydrate modifications were evident on either protein and subcellular fractionation studies revealed an endoplasmic reticulum localization. The degradation rates of both aberrant enzymes were similar, suggesting that both were degraded by a similar mechanism within the endoplasmic reticulum.

Protein Degradation in the Cytoplasm

Proteinase E is the *S. cerevisiae* equivalent of the mammalian proteasome (Achstetter et al., 1984a,b), a multifunctional enzyme complex responsible for stress-induced proteolysis of cytoplasmic components (Heinemeyer et al., 1991), for degradation of abnormal proteins (Egner et al., 1993), and probably for degradative catabolite inactivation of at least some glucose-sensitive enzymes, e.g., phosphoenolpyruvate carboxykinase (Burlini et al., 1989), malate dehydrogenase (Funagama et al., 1985), and possibly fructose-1,6-bisphosphatase, though this is controversial (Teichert et al., 1989; Schork et al., 1994; Chiang and Schekman, 1991). The proteasome has been highly conserved from yeast to man and is composed of 12 to 14 subunits of between 20 to 35 kDa comprising an enzyme complex of a total of about 700

kDa forming a hollow cylinder which displays a variety of proteolytic activities. Heinemeyer et al. (1991) isolated two complementation groups (PRE1; PRE2) which are defective in the "chymotrypsin-like" activity of the *S. cerevisiae* proteasome. The PRE1 gene is essential for life and encodes a 22.6 kDa protein with significant homology to proteasome subunits of the rat and *Drosophila*. Diploids homozygous for the temperature-sensitive allele *pre1-1* are defective in sporulation and under nutritional and temperature stress conditions exhibit decreased protein degradation and accumulate ubiquitin-protein conjugates.

In order to ensure that the degradation of cytosolic proteins is limited to a specific subset of those present, the proteins to be degraded must be targeted to the proteasome in some way. The ubiquitin-linked proteolytic pathway serves this purpose in organisms as diverse as mammals and yeast (Finley and Chau, 1991). Proteins to be degraded are multi-ubiquitinated on specific lysine residues (Chau, 1989; Johnson et al., 1992). Similarly to the results for proteasome mutants discussed above, *S. cerevisiae* mutants which are unable to ubiquitinate proteins either due to the inability to express ubiquitin (Finley et al., 1987) or are unable to express ubiquitin conjugating enzymes (E2s; Seufert and Jentsch, 1990) are susceptible to stress. The fact that proteasome mutants exhibit decreased proteolysis rates and accumulate ubiquitin-protein conjugates under stress conditions (Heinemeyer et al., 1991) and decreased degradation of proteins which are subject to ubiquitin-mediated degradation in wild type cells

(Seufert and Jentsch, 1992), strongly suggest that the proteasome is part of the ubiquitin system which is responsible for a substantial portion of cytosolic protein degradation.

The glucose effect occurs in yeast cells following addition of glucose to cultures which were previously growing in media containing an unrelated carbon source. In these cases glucose catabolites act to transcriptionally repress the synthesis of certain enzymes (catabolite repression) and also to induce the inactivation (by degradation or otherwise) of certain enzymes already present (catabolite inactivation; Holzer 1976; Holzer and Purwin, 1986). As was noted above, some controversy exists regarding the site of degradation of proteins undergoing catabolite inactivation as during adaptation to glucose (see Schork et al., 1994 and response of H.-L. Chiang and R. Schekman following). This debate is based on the use of vacuolar and proteasomal mutants to determine the site of degradation. Vacuolar mutants have been used in several cases to indicate that certain glucose-sensitive enzymes are not degraded in the vacuole (see above; Burlini et al., 1989; Funagama et al., 1985; Chiang and Schekman, 1991). But, in the case of fructose-1,6-bisphosphatase, one laboratory reports that a proteasome mutant was unable to degrade it while vacuolar mutants could (Teichert et al., 1989; Schork et al., 1994). In another laboratory (Chiang and Schekman, 1991) vacuolar mutants were unable to degrade this protein in response to glucose. These investigators suggested that the proteasome mutants used by the former group exhibited a pleiotropic phenotype and

therefore are not reliable, and Chiang and Schekman are doing further work to settle the matter (response to Schork et al., 1994). The role of the proteasome in degradation in yeast is not well defined at this point in time due to a relative lack of research on this system compared to the huge amount of research which has been conducted on the vacuole.

Protein Degradation in the Vacuole

The majority of the work done regarding the degradative role of the vacuole to date has involved the investigation of the proteases which are resident and their proteolytic specificities. This has left the questions of how and which proteins and organelles enter the vacuole largely unanswered. As with the mammalian lysosome, the major routes of entry into the vacuole are via the secretory pathway (biogenesis), via endocytosis, and via autophagy. Biogenesis has already been discussed; consequently this section shall mainly deal with endocytosis and autophagy.

Endocytosis. Internalization of alpha-factor (aF), one of the two peptide hormones responsible for synchronizing mating between the two cell types of *S. cerevisiae*, and its receptor serves as the model system for endocytosis in yeast. aF receptor is endocytosed constitutively when not bound to its ligand and in a regulated manner when bound to aF (Davis et al., 1993). Normal rates of aF receptor internalization is dependent upon clathrin, suggesting internalization via clathrin coated pits as commonly occurs with this class of G protein-coupled receptors in higher eukaryotes (Tan et al., 1993). After internalization, aF bound

to its receptor proceeds via vesicular intermediates (Raths et al., 1993; Davis et al., 1993; Singer and Reizman, 1990; Schimmoller and Reizman, 1993) through early and late endosome compartments (Davis et al., 1993; Wichman et al., 1992; Schimmoller and Reizman, 1993). The early endosome may be the site of convergence of the vacuolar biogenesis and endocytic pathways (see Fig. 1-2; Vida et al., 1993; Davis et al., 1993) where vacuolar and endocytosed proteins transiently reside in identical compartments. Transport between endosomal compartments is dependent on YPT7, a ras-like small guanine nucleotide-binding protein (Wichmann et al., 1992; Schimmoller and Reizman, 1993). Finally, aF is delivered to the vacuole where it is degraded (Wichmann et al., 1992).

Autophagy. Eukaryotic cells possess specific systems for degrading their own proteins and organelles when they become superfluous, or have deteriorated, or when the cell is under stress and requires substrate for new protein synthesis and organelle biogenesis. Autophagy is the process whereby the lysosome/vacuole takes up cellular components either by surrounding portions of the cytoplasm within invaginations of its membrane and assimilating and degrading the contents (microautophagy; Ahlberg et al., 1985; Mortimore et al., 1988) or by fusing with and assimilating the contents of autophagosomes (or autophagic vacuoles) in which portions of cytoplasm have been sequestered (macroautophagy; Dunn, 1990a,b; Takeshige et al., 1992; Baba et al., 1994). In mammalian cells, autophagic vacuoles are formed in response to amino acid

deprivation in perfused rat livers when portions of the rough endoplasmic reticulum (RER) lose their ribosomes, pinch off from the main body of the RER, and surround portions of the cytoplasm sequestering the contents from the remainder of the cytoplasm (Dunn, 1990a). The autophagic vacuoles formed may contain ribosomes, mitochondria, peroxisomes and soluble proteins. The newly formed autophagic vacuoles rapidly become acidic, possibly by acquiring vacuolar H^+ ATPase complexes by fusion with vesicles containing them derived from the Golgi apparatus (Dunn, 1990b). The autophagic vacuole then acquires hydrolytic enzymes by fusing with primary lysosomes and thereby matures into a degradative autolysosome (Dunn, 1990b). Macroautophagy in yeast has not been so well characterized but analysis of autophagy mutants should soon yield more data on this process (Tsukada and Ohsumi, 1993; Thumm et al., 1994) and will be discussed in the next section.

Nonselective autophagy. Autophagy is considered by many to be mainly responsible for a nonselective bulk turnover of proteins which occurs at the site for nonselective degradation, i.e., the lysosome/vacuole, in response to nutritional or developmental stress (Takeshige, et al., 1992; Simeon et al., 1992; Mortimore and Pösö,1987; Knop et al., 1993; Ahlberg et al., 1985; Jones, 1991a; Kopitz et al., 1990). Kopitz et al. (1990) used electrodisruption of the plasma membrane of isolated hepatocytes to deplete cells of their cytosol and soluble proteins to form "cell corpses" which still contain their organelles, including autophagosome. In this study seven proteins for which normal half-lives where

calculated and which ranged from 0.9 h to 17.4 h were found to be sequestered into isolated autophagosomes at the same rate in the presence of inhibitors of lysosomal proteolysis. The authors concluded that the autophagic-lysosomal system operates nonselectively.

Evidence also exists for nonselective uptake of cytoplasmic components by autophagy in *S. cerevisiae*. Utilizing mutants strains of *S. cerevisiae* which contained null mutant copies of the genes for the vacuolar endoproteases PrA and PrB, it was found that the vacuole is responsible for about 40% of the proteolysis which occurs during logarithmic growth but this increases to 85% during starvation for nitrogen (Teichert et al., 1989). After 24 h of nitrogen starvation, 45% of all cellular proteins had been degraded in the vacuole. Cytosolic proteins which are normally degraded independently of the vacuole, e.g., enzymes of the gluconeogenic pathway (fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase) were found to accumulate in the vacuoles of nutrient deprived vacuolar mutants (Egner et al., 1993). Coincident with the accumulation of cytosolic proteins in the vacuole there was an accumulation of vesicular bodies within the vacuolar lumen, suggesting that cytoplasmic contents are being taken up into the vacuole by autophagy but are not degraded because of the lack of protease activity in vacuolar mutant cells (Egner et al., 1993; Simeon et al., 1992).

Ohsumi and his colleagues (Takeshige et al., 1992; Baba et al., 1994) have been studying autophagy in *S. cerevisiae* by rendering the vacuole

proteolytically inactive either by utilizing strains lacking PrA and PrB or by treating normal cells with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). As a result of depriving the cells of nitrogen, carbon, or essential amino acids, "autophagic bodies" were seen to accumulate in the vacuoles of cells with inactivated vacuoles. The concentrations of three cytosolic marker enzymes, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and phosphoglycerate kinase, were present in the autophagic bodies at the same concentration as in the cytosol but were not present in the vacuolar sap. The autophagic bodies also contained ribosomes, mitochondria, RER, and glycogen and lipid granules at approximately the same density as in the cytoplasm. Using a freeze-substitution method of fixation for transmission electron microscopy which preserves yeast ultrastructure remarkably well, spherical structures bound by double membranes were observed in the cytoplasm near the vacuole which contained cytoplasmic components and had a similar appearance to autophagic vacuoles seen in mammalian systems (Dunn, 1990a,b). These autophagic vacuoles were also observed with their outer membranes continuous with the vacuolar membrane as if fusing with the vacuoles and depositing the autophagic bodies into the vacuolar lumen.

Taken together, these results strongly suggest that nutrient deprivation in *S. cerevisiae* initiates a process whereby random portions of the cytosol are surrounded by a double membrane of unknown origin forming autophagic vacuoles. These autophagic vacuoles then fuse with and deposit their contents

into the vacuole where they are degraded to provide substrates for new synthesis which will allow the cells to adapt to prevailing nutrient conditions and remain viable.

Selective autophagy. A review of the mammalian literature reveals several interesting instances of selective autophagy. When fed clofibrate (an anti-hyperlipidemia drug), rats strongly induce peroxisomes containing the enzymes catalase and acyl-CoA oxidase (Luiken et al., 1992). In three separate experiments, hepatocytes were isolated from rats which had been fed clofibrate and then incubated in the absence of amino acids for 4 h to induce the autophagic pathway. The levels of peroxisomal and cytosolic proteins before and after incubation were determined. The relative levels of both peroxisomal enzymes were ~60% after incubation when compared to 0 h levels; this was significantly lower than the cytosolic marker enzymes levels which were all in the range of 79 to 89% of initial. Co-incubation with 3-methyl-adenine, an inhibitor of autophagy, or long-chain fatty acids, which are substrates for acyl-CoA oxidase, blocked the decrease in peroxisomal enzymes while short-chain fatty acids, not substrates for acyl-CoA oxidase, did not. These data indicate that peroxisomes are specifically degraded in cases in which the enzymes they contain are not needed (i.e., when incubated without acyl-CoA substrates).

Patients with cerebro-hepato-renal Zellweger syndrome and cultured fibroblasts from patients with this genetic defect have few normal peroxisomes; instead membrane structures referred to as peroxisomal ghosts form. These

contain some peroxisomal membrane proteins but lack normal peroxisomal matrix contents (Santo et al., 1988; Arias et al., 1985). Apparently, the lesion in this disease is a defect in peroxisomal biogenesis in which certain peroxisomal matrix enzymes are mislocalized to the cytosol and rapidly degraded, instead of transported into the peroxisomes, (Tager et al., 1986). Heikoop et al., (1992) used double labeling immunoelectron microscopy on Zellweger fibroblasts to show that most peroxisomal ghosts in fact contain lysosomal hydrolases. Treatment with the autophagy inhibitor 3-methyl-adenine caused an increase in the number of morphologically normal peroxisomes, suggesting that Zellweger peroxisomes are selectively degraded by autophagy.

There has been extensive study of the fate of phenobarbital-induced smooth endoplasmic reticulum (SER) and the resident detoxifying enzymes cytochrome P450 and NADPH-cytochrome P450 reductase upon withdrawal of the drug. Bolender and Weibel (1973) showed morphometrically that there was a concurrent increase in the total volume (800%) and number (96%) in autophagic vacuoles and a preferential removal of SER membranes. Masaki et al. (1984) showed a decrease in the cellular content of SER enzymes and a concurrent increase in the number autophagic vacuoles after phenobarbital withdrawal. The same group directly showed, using quantitative immunoblots and immunoelectron microscopy, that autophagic vacuoles isolated from liver cells recovering from phenobarbital treatment contain large amounts of the SER

enzymes cytochrome P450 and NADPH-cytochrome P450 reductase (Masaki et al., 1987).

These data show that in certain nutritional, pathological, and pharmacological situations selective autophagic degradation can be induced in higher eukaryotes. As discussed above, several instances of selective, degradative "catabolite inactivation" have long been recognized as occurring in *S. cerevisiae* (Holzer 1976; Holzer and Purwin, 1986). Of the enzymes known to undergo catabolite inactivation, there is evidence for only one being degraded in the vacuole (Chiang and Schekman, 1991) and even this is controversial (Schork et al., 1994). Therefore, little is known about how a soluble protein might enter the vacuole. It has been suggested that a certain protein(s) which traverses the secretory pathway may serve as a receptor or channel to allow soluble protein entry into the vacuole (Chiang and Schekman, 1991).

The methylotrophic yeasts have served as a model system both for peroxisome biogenesis and degradation due to the ease of peroxisome induction and detection in this system (see discussion above in "Methanol Metabolism and Peroxisomes in Yeast"). Bormann and Sahm (1978) were the first to note that methanol-induced cells of the methylotrophic yeast *Candida boidinii*, containing high levels of the peroxisomal enzymes alcohol oxidase and catalase, exhibited a marked decrease in enzyme activity and number of peroxisomes when subsequently incubated in a new carbon source (ethanol). It was observed that the degradation of peroxisomes stimulated by ethanol did not

require protein synthesis. Later experiments by Hill et al. (1985) confirmed the fact that the loss of peroxisomal enzyme activity was due to degradation of the protein and not by limited or reversible inactivation. Electron microscopic examination of cells undergoing ethanol adaptation revealed that peroxisomal enzyme degradation was due to degradation of the entire peroxisomes and not to certain enzymes within the peroxisome. Together these data indicate that methanol-induced peroxisomes in *C. boidinii* are degraded by autophagy upon addition of ethanol to the media.

The fate of methanol-induced peroxisomes has been most carefully studied in *Hansenula polymorpha*. Veenhuis et al. (1978) added glucose to a final concentration of 0.4% to stationary methanol-grown cultures and determined that alcohol oxidase and catalase activities decreased precipitously over the ensuing 4 h to 22% and 34% of the values at 0 h, respectively. Morphometric evaluation of transmission electron micrographs of cells before and after 4 h of glucose adaptation revealed a decrease in the volume occupied by peroxisomes of 92%. These authors concede that their data do not prove that the peroxisomes and enzymes disappear due to degradation since they were measuring enzyme activity and not protein and their system could not take possible changes in peroxisome synthesis into account. Their morphometric analyses did not reveal the cause of the disappearance of the peroxisomes and diluting out among new daughter cells could not be ruled out.

The same laboratory (Bruinenberg et al., 1982) investigated the phenomenon of glucose-mediated catabolite inactivation in *H. polymorpha* further, using HPLC to show that indeed AOX and catalase protein are being depleted at approximately the same rates as enzyme activity during adaptation to glucose. Switching methanol-induced *H. polymorpha* cells to media lacking any carbon source had minimal effects on AOX and catalase activity and protein levels, suggesting that the loss of protein is directly mediated by glucose or its metabolites. Again, synthesis effects were not evaluated so it could not be determined whether peroxisome degradation was enhanced or whether it remained the same and peroxisome synthesis had decreased. An interesting phenomenon was noted when methanol-induced cells were switched to glucose media which also contained 6 mM KCN; in this case, AOX activity fell very rapidly (80% loss in 1 h) but AOX protein levels remained the same for at least 4 h. They were able to account for this phenomenon by noting that the time course for activity loss corresponded almost exactly with decreases in AOX-bound FAD. It appears that KCN mediates a loss of the FAD prosthetic group which renders the enzyme largely inactive.

A detailed ultrastructural description of the events that occur in *H. polymorpha* when methanol-induced cells are switched to glucose was prepared by this group (Veenhuis et al., 1983). The earliest change that was noted was the appearance of a variable number (2 to 12) of layers of electron-dense membranes surrounding individual peroxisomes within a cluster which appeared

to sequester a given peroxisome from the cytosol into an autophagosome. Other cytoplasmic components were never observed together with peroxisomes in an autophagosome as determined by transmission electron microscopic surveys of glutaraldehyde/OsO_4 or $KMnO_4$ fixed cells or isolated autophagosomes. The authors suggested that the limiting membranes of the autophagosomes arose *de novo* since they saw no evidence for enwrapping of peroxisomes by existing membrane components. Notwithstanding this fact, Veenhuis and colleagues had earlier noted that methanol-induced peroxisomes where almost always seen in close juxtaposition to endoplasmic reticulum cisternae (Veenhuis et al., 1978). Therefore, it can not be ruled out that in *H. polymorpha*, as in higher eukaryotes (Dunn 1990a), organelles may be sequestered from the cytosol by segments of endoplasmic reticulum.

Similarly to rat liver autophagosomes (Dunn 1990a,b), the early autophagosomes of *H. polymorpha* cells undergoing catabolite inactivation were devoid of vacuolar hydrolases and then acquired them as they matured (Veenhuis et al., 1983). Hydrolases were observed to be procured by two different mechanisms: 1) extensions of the limiting membranes of the autophagosome protruded into a nearby vacuole, surrounding a portion of the vacuole and incorporating that portion of the vacuole into the thereby newly formed autolysosome; or 2) autophagosomes were seen to fuse with vacuoles and deposit their contents (a peroxisome) into the vacuolar lumen where the

peroxisomes were degraded. The authors noted that the molecular mechanisms behind these actions remain unknown.

One of the events that must occur if peroxisomes are to be selectively degraded is that they must be recognized as destined for degradation. Evidence to support this hypothesis is provided by van der Klei et al. (1991) who studied the fate of AOX in *H. polymorpha* peroxisome assembly mutants (PER mutants). One class of these mutants does not grow in methanol but develops very large AOX crystalloids in the cytosol which are not bounded by peroxisomal membranes. When glucose is added to methanolic cultures of this mutant class, they begin to grow but the AOX crystalloids are not degraded or seen to be taken up into the vacuole, and AOX activity and protein remain unchanged during a 4 h time course. In the wild type *H. polymorpha*, as shown in the previous studies already mentioned, AOX activity and protein and peroxisomes were > 50% depleted in 4 h of glucose adaptation. This suggests that the peroxisomal membrane mediates the degradation of AOX: Presumably molecules in the peroxisomal membrane allow the peroxisomes to be recognized by the membranes which form the limiting membranes of autophagosomes. In contrast to glucose, ethanol caused a rapid decrease in AOX activity in PER mutants which form cytosolic crystalloids, decreasing AOX activity levels by ~50% in 4 h (van der Klei et al., 1991). Immunoblot analysis of AOX during ethanol adaptation of PER mutants revealed that AOX protein levels did not diminish during this time, suggesting "modification inactivation" rather than

degradation. This result may be explained by a loss of AOX-bound FAD which has previously been shown to inactivate the protein (Veenhuis et al., 1983). The mechanism of inactivation was not determined in this study (van der Klei et al., 1991) but cytochemical analysis revealed that AOX crystalloids which had lost activity during ethanol treatment had not been taken up into the vacuole but, rather, remained in the cytosol.

Autophagy mutants. While examples of autophagy have been reported in yeast, still very little is understood about the mechanisms of autophagic sequestration of specific substrates, and the field has remained largely descriptive. Only very recently has the power of yeast genetics been brought to bear on the problem of autophagy, and important details regarding the molecular mechanisms of autophagy should be forthcoming.

The first autophagy-defective mutants in yeast were produced in vacuolar proteinase-deficient strains of *S. cerevisiae* (Tsukada and Ohsumi; 1993). It has been noted that starvation for nitrogen, essential amino acids, or carbon produced an increase in nonselective protein degradation mediated by the vacuoles of normal yeast (Teichert et al., 1989). Correspondingly, starvation caused an accumulation of autophagic bodies in the vacuoles of cells with defective PrA and/or PrB, i.e., with proteolytically inactive vacuoles (Takeshige et al., 1992; Baba et al., 1994). Tsukada and Ohsumi (1993) chemically mutated a *S. cerevisiae* strain lacking both PrA and PrB and then looked for vacuolar accumulation of autophagic bodies by light microscopy. Clones that were

unable to sequester cytoplasmic components into the vacuole were readily identifiable in this way (no accumulation in the vacuole during starvation) and were classified APG mutants. Further characterization of a clone designated a*pg1-1* revealed that it was sensitive to nitrogen deprivation, i.e., it became inviable after 2 d in nitrogen-free media as indicated by uptake of phloxine B (red colonies) when plated onto agar plates containing this dye. Thereafter nitrogen deprivation sensitivity was used as an initial selection to aid in the screening for further mutants and 75 recessive a*pg* mutants were assigned to 15 complementation groups. It was determined that the vacuoles of these mutants were normal for functions other than autophagy, e.g., they were acidic and they were able to accumulate endocytosed substances (Tsukada and Ohsumi, 1993). Therefore, it was concluded that the defects being studied in these yeast were not caused by a generalized defect within the vacuole itself.

Egner et al. (1993) showed that when one of the two subunits of the cytosolic protein fatty acid synthase is overexpressed in starved protease mutant *S. cerevisiae*, then the excess, unassembled subunits accumulate in the vacuole, along with other cytosolic proteins. In contrast, in fed cells, the unassembled subunits are degraded in the cytosol by the proteasome. Since the vacuole is the site of nonselective degradation during starvation, the diversion of unassembled subunits from their normal site of degradation to the vacuole was taken as evidence that they are taken up into the vacuole by a nonselective bulk process. These same investigators took advantage of this

nonselective accumulation as a marker for autophagy to screen for autophagy mutants (AUT) using a colony screening procedure (Thumm et al., 1994). Chemically mutagenizing PrA/PrB deficient strains which also lacked a functional gene for one of the two fatty acid synthase subunits (e.g., α) and then using a direct colony immunoassay for the other fatty acid synthase subunit (e.g., β), they screened for clones which were not able to accumulate the appropriate fatty acid synthase subunit in the vacuole. This rather time-consuming process (5 to 9 d for screening) has so far yielded 3 complementation groups.

Analysis of the proteins encoded by the 18 genes found to affect autophagy which have been isolated to date should yield the first information on the molecular mechanisms of nonselective autophagy. Autophagy has only recently been acknowledged to occur in yeast. In that short time, more progress has been made than in the several decades that has been expended in the study of mammalian autophagy. Now that the advantages of yeast cell biology and genetics are being utilized in regards to autophagy, our new knowledge of the mechanisms of autophagy should enable steady progress both in yeast and in higher eukaryotes, if homologous genes are found to act in these diverse organisms, as has proven to be the case for secretion, nuclear transport, protein sorting and other areas of basic cell function.

Genetic Analysis in Methylotrophic Yeasts

As must be obvious from the foregoing discussions, *Saccharomyces cerevisiae* is by far the best studied of the yeasts both in terms of cell and molecular biology; there are hundreds of mutant strains available to aid one's studies and straightforward strategies have been worked out for the molecular genetic manipulation of the *S. cerevisiae* genome. In most cases this renders *S. cerevisiae* the clear choice for mutagenesis and gene cloning strategies. One case contrary to this rule is that of the study of selective autophagy, a phenomenon not known to exist in *S. cerevisiae* but very easy to manipulate in the methylotrophic yeasts *P. pastoris* and *H. polymorpha*. Recent developments have made these yeast more amenable to cell biological and genetic studies.

Classical Genetics

Two of the genera of methylotrophic yeasts, *Pichia* and *Hansenula* are ascomycetous: their life cycles are characterized by genetically defined stages, i.e., haploid and diploid (Gleeson et al., 1984; Cregg, 1987; Gleeson and Sudberry, 1988; Gould et al., 1992). The life cycles of *P. pastoris* and *H. polymorpha* are very similar to that of *S. cerevisiae* in that they can be maintained indefinitely in the haploid vegetative state and by changing the composition of their growth media they can be forced to mate and become diploid and remain vegetative in this state if desired. Changing the media again causes the diploids to undergo meiosis and haploid spores result which can germinate in favorable media and resume haploid vegetative growth. Also like

S. cerevisiae, these yeasts exist in one of two mating types. Mating type switching occurs under poor nutritional conditions so these yeasts are homothallic, and mating can be caused to occur between any two strains.

These features just mentioned allow the routine use of backcrossing and complementation analysis. These invaluable techniques are a large part of the reason that species that can be maintained in the haploid state are such powerful systems for the identification of new genes. A prolonged haploid state makes the consequences of recessive mutations easily detectable. Backcrossing makes it possible for mutant strains to be refined so that they carry only one mutant gene relevant to the phenotype of interest, and complementation analysis allows the phenotypic identification of complementation groups or genes.

Molecular Biology

Development of the methylotrophic yeasts *P. pastoris* and *H. polymorpha* as host systems for DNA transformations has been taken place over the past decade (Cregg et al., 1985; Tikhomirova et al., 1986, 1988; Roggenkamp et al., 1986; Gleeson et al., 1986). As it turns out *P. pastoris* is more amenable to transformation, yielding transformation rates of ~10^4 transformants/ μg DNA, similar to that obtainable in *S. cerevisiae* but 1 to 2 orders of magnitude higher than has been obtained in *H. polymorpha* (J.M. Cregg, personal communication). Due to the fact that the AOX promoter of *P. pastoris* is tightly regulated, i.e., tremendously induced in the presence of methanol and is almost

completely turned off in its absence, this yeast has been developed as a host system for the production of foreign proteins (Cregg and Madden, 1988; Wegner, 1990; Sreekrishna et al., 1988) , e.g., the secreted isoform of the Alzheimer's amyloid beta-protein precursor (Wagner et al., 1992), *Bordetella pertussis* pertactin (Romanos et al., 1991), mouse epidermal growth factor (Clare et al., 1991), bovine lysozyme (Brierly et al. 1990), and human tumor necrosis factor (Sreekrishna et al., 1989). Reports of as much as 3 g of protein/liter of culture were caused to be secreted into the media.

A number of genes have been cloned and sequenced in *P. pastoris*, particularly ones whose products are active in the peroxisome biogenesis pathway (Koutz et al. 1989; Ellis et al., 1985; Spong and Subramani, 1993; Crane et al., 1994; McCollum et al., 1993; Gould et al., 1992). To accomplish this, certain tools or reagents have to be available and have been developed in at least two separate labs: a set of auxotrophic strains with essentially wild type genetic background; plasmids that act as *E. coli--P. pastoris* shuttle vectors, (those developed have been based on the plasmid pBR322) containing the ampicillin resistance gene, an *E. coli* origin of replication, *P. pastoris* autonomous replication sequences and an auxotrophic selectable marker, e.g., the histidine dehydrogenase gene from either *S. cerevisiae* or *P. pastoris*; and genomic DNA libraries for the isolation of *P. pastoris* genes by functional complementation of mutants or by nucleic acid hybridization (Gould et al., 1992; Cregg et al., 1985).

With these tools the molecular cell biology of *P. pastoris* can be probed and the molecular mechanisms of processes for which methylotrophic yeasts are good models, e.g., peroxisome biogenesis and degradation, are beginning to be elucidated.

CHAPTER 2
MATERIALS AND METHODS

Yeast Strains and Media

The essentially wild type methylotrophic yeasts used in these studies were a leucine auxotrophic (leucine$^-$) strain of *Hansenula polymorpha* (A16, a backcross derivative of strain L1 (Gleeson et al., 1986); a histidine auxotroph (histidine$^-$) of *Pichia pastoris* (GS115) and an arginine auxotroph of *P. pastoris* (GS190-3). These strains were all the very generous gifts of J.M. Cregg. The strains of *P. pastoris* lacking proteinase A (PrA$^-$) and/or proteinase B (PrB$^-$) are all histidine auxotrophs: SMD1163 (PrA$^-$, PrB$^-$); SMD1165 (PrB$^-$); and SMD1168 (PrA$^-$). These strains were kindly donated by L.V. Benningfield of the Phillips Petroleum Co. Licensing Office as part of the Pichia Yeast Expression System.

TABLE 2-1. Yeast strains

STRAIN (SPECIES)	GENOTYPE	PHENOTYPE
A16 (*H. polymorpha*)	*leu2*[a]	Leucine$^-$
GS115 (*P. pastoris*)	*his4*	Histidine$^-$
GS190-3 (*P. pastoris*)	*arg4*	Arginine$^-$
SMD1163 (*P. pastoris*)	*his4, pep4, prb1*	Histidine$^-$, PrA$^-$, PrB$^-$
SMD1165 (*P. pastoris*)	*his4, prb1*	Histidine$^-$, PrB$^-$
SMD1168 (*P. pastoris*)	*his4, pep4*	Histidine$^-$, PrA$^-$
WDY1[b] (*P. pastoris*)	*his4, gsa1-1*	Histidine$^-$, GSA1$^-$
WDY2[b] (*P. pastoris*)	*his4, gsa2-1*	Histidine$^-$, GSA2$^-$
WDY3[b] (*P. pastoris*)	*his4, gsa2-2*	Histidine$^-$, GSA2$^-$

[a] See text for details of nomenclature
[b] Strains developed during the course of this study

The media utilized for the growth of yeast in this study consist of the following:

MIM (methanol induction medium): 6.7 g/L yeast nitrogen base without amino acids (Difco), 0.5% methanol, 40 mg/L histidine or arginine, and 40 μg/L biotin.

GA (glucose adaptation medium): 6.7 g/L yeast nitrogen base without amino acids, 2.0% glucose, 40 mg/L histidine or arginine, and 40 μg/L biotin.

EA (ethanol adaptation medium): 6.7 g/L yeast nitrogen base without amino acids, 0.5% ethanol, 40 mg/L histidine or arginine, and 40 μg/L biotin

YPD: 1% yeast extract, 2% bactopeptone (both from Difco), 2% dextrose

SM (sporulation medium): 0.5% sodium acetate, 1.0% KCl, 1.0% dextrose

DSM (diploid selection medium): 6.7 g/L yeast nitrogen base without amino acids, 2.0% glucose, and 40 μg/L biotin.

Transformation plates: 6.7 g/L yeast nitrogen base without amino acids, 2.0% glucose, 1 M sorbitol, and 40 μg/L biotin.

LSM (low sulfate medium): autoclave stock solution of 10x salts: 6 g/L $MgCl_2 \cdot 6H_2O$, 1.23 g/L K_2HPO_4, 8.77 g/L KH_2PO_4, 5 g/L NaCl, 10 g/L NH_4Cl; autoclave stock solution of 1 M $CaCl_2$; autoclave stock solution of 100 mg/L $FeCl_3 \cdot 6H_2O$; to prepare working solution of LSM mix 0.1 g yeast extract in 10 mL 10x salts and bring to 100 mL with water, pH to 5.8 and autoclave; after cooling add 0.9 mL $CaCl_2$ and 0.5 mL $FeCl_3$; supplement with amino acids and carbon source as needed.

Enzyme Assays

Preparation of cell-free extracts of yeast was accomplished by removing 2mL samples of cultures to culture tubes on ice at appropriate times and harvesting cells by centrifugation at 1000 x g, 4°C for 1 min and aspirating the supernatant; 1 mL of a buffer containing 20 mM Tris/Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, was added to the cell pellet and phenylmethylsulfonyl fluoride to a

final concentration of 1 mM was added while mixing just prior to preparation of extract. Approximately 0.5 mL of 0.5 mm diameter acid-washed glass beads were added and then the tubes were vortexed 2 x 1min, placing tubes on ice ≥ 1 min between each occasion of vortexing. The tubes were then centrifuged 1000 x g, at 4°C for 6 min, and the supernatants carefully aspirated and placed in 1.5 mL microfuge tubes on ice.

Formate dehydrogenase (FDH) was assayed utilizing sodium formate as a substrate and following the reduction of NAD^+ at 340nm according to the procedures of Kato (1990).

Measurement of alcohol oxidase (AOX) activity was performed with methanol as a substrate, producing hydrogen peroxide, in turn metabolized by horseradish peroxidase (HRP) to oxidize 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS). The generation of oxidized ABTS was followed spectrophotometrically at 410 nm (Sahm and Wagner, 1973).

Proteinase A (PrA) assays were performed according to Jones (1991b) and direct colony assays for carboxypeptidase Y (CPY) activity were done according to Jones (1977).

Preparation of Antisera

Formate dehydrogenase purified from *Candida boidinii* (100 μg; Sigma Chemical Co.) was emulsified in Freund's adjuvant and injected intradermally into a rabbit. A boost injection containing 50 μg FDH emulsified in Freund's

adjuvant was administered intradermally. The immunological properties of FDH from *C. boidinii* and *P. pastoris* have been shown to be quite similar, even though the subunit size is somewhat larger in *P. pastoris* (Hou et al., 1982). Immunological analysis was performed on cell-free extracts which were electrophoresed on 7.5% SDS-polyacrylamide gels and then transferred to nitrocellulose. Immunoblotting was performed according to the ECL method of Amersham (Arlington Heights, IL). On immunoblots, a single band of M_r $45x10^3$ was recognized in methanol-induced cellular extracts of *P. pastoris* (Tuttle and Dunn, *in press*). This band is in close agreement with that reported for FDH subunits of *P. pastoris* (Hou et al., 1982). Preabsorption of the antiserum with purified FDH greatly diminished the signal on immunoblots. Preabsorption with the same amount of an unrelated protein (AOX) had little effect on immunostaining (data not shown).

Purified AOX from *P. pastoris* (104 µg; Sigma Chemical) was emulsified in Freund's adjuvant and injected into a lymph node of a rabbit. Boost injections were administered subcutaneously at 6 wk intervals that included 52 µg of antigen in Freund's adjuvant. On Western blots, the antiserum recognized a single protein of 74 kDa in total cellular extracts of both *P. pastoris* and *H. polymorpha* only when grown under peroxisome-induction conditions (Tuttle and Dunn, *in press*). Immunoelectron microscopic analysis of methanol-induced yeast displayed strong, specific localization limited to the crystalloid core of giant peroxisomes (see Fig. 2-1).

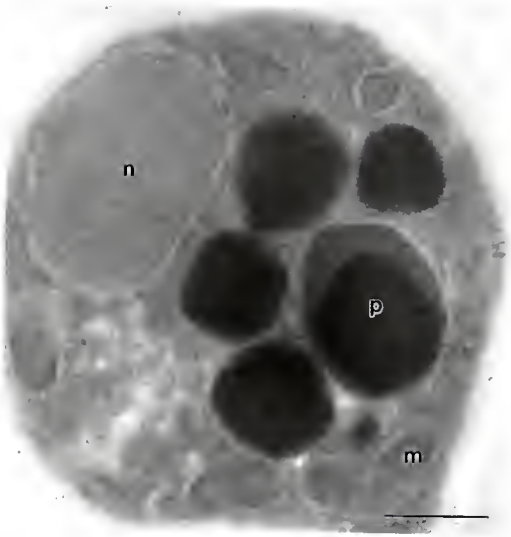

Figure 2-1. Immunoelectron microscopic localization of alcohol oxidase in methanol-induced *P. pastoris*. Wild type *P. pastoris* cells (GS115) were grown to stationary phase then fixed, embedded, and subjected to immunoelectron microscopic analysis utilizing alcohol oxidase antisera. AOX was localized with 10 nm gold conjugated to goat anti rabbit antibodies. p = peroxisomes m = mitochondrion; n = nucleus; bar = 0.5 μm.

Electron microscopy

Ultrastructural analysis was performed on potassium permanganate-fixed cells in which membrane profiles are effectively delineated in methylotrophic yeasts (Veenhuis et al., 1983). Cells were harvested from a 1 mL of culture sample by centrifugation for 1 min at 15,000 x g at room temperature, washed in 1 mL water, and fixed in 1 mL 1.5% $KMnO_4$ in veronal-acetate buffer (0.3 mM sodium acetate; 0.3 mM sodium barbital, pH 7.6) for 20 min at room temperature. The specimens were dehydrated in increasing concentrations of ethanol, ending in absolute, followed by 100% propylene oxide then infiltrated with a 50:50 mixture of propylene oxide and (Polysciences, Inc., Warrington, PA) for 2 d. The samples were pelleted at 15,000 x g and then resuspended in fresh POLY/BED 812 and placed under vacuum overnight then polymerized in a 60°C oven for 24 h. The blocks were sectioned on a Reichert UltraCut E microtome (Cambridge Inst.; Deerfield, IL) by Denny Player and examined on a JEOL 100CX II transmission electron microscope.

Immunoelectron microscopic localization of alcohol oxidase was performed on glutaraldehyde-fixed, POLY/BED 812-embedded cells as described by Clark (1991). To block non-specific binding of antibodies the sections were incubated in 8% bovine serum albumin (BSA) in TBS then incubated in 1:100 rabbit anti-alcohol oxidase serum in TBS + 0.1% BSA for 1 h at room temperature in a moistened chamber. After washing in 0.1% BSA in TBS, grids were incubated in a 1:30 dilution of protein A conjugated to 10 nm

colloidal gold (Amersham, UK) for 1 h to localize antigen/antibody complexes followed by washing in TBS.

Protein Synthesis and Degradation

Synthesis of Peroxisomal Proteins

Single colonies from YPD plates were pre-cultured in low sulfate medium (Daum et al., 1982) containing 30 mg/L leucine or 20 mg/L histidine, and 2% glucose. Pre-cultures were inoculated into low sulfate medium with 0.5% methanol and then incubated until the desired growth phase had been attained. Methanol-induced exponential and stationary phase cells and stationary phase cells to which glucose had been added were labeled for 15 min with [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN Biomedicals, Irvine, CA, USA). Cell-free extracts were made by vigorous vortexing of harvested cells in the presence of glass beads. Total cellular proteins were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970), stained with 1.0% Coomassie blue, and the bands corresponding to the methanol-induced peroxisomal enzymes dihydroxyacetone synthase (DHAS) and alcohol oxidase (AOX) were excised from the gels (see Fig. 3-1). The gel pieces were solubilized in a scintillation cocktail composed of 0.54% PPO, 0.0135% dimethyl POPOP in a solution of 90% toluene and 10% TS-1 Solubilizer (Research Products International, Inc., Mt. Prospect, IL, USA) and radioactivity quantified using a Beckman LS5000TD scintillation counter. In

some cases, the labeled proteins were electrophoretically transferred to nitrocellulose and their relative amounts quantified from an autoradiograph by laser densitometry.

Degradation of Peroxisomal and Mitochondrial Proteins

Single colonies from YPD plates were precultured in low sulfate medium containing 2% glucose for 24 h. The cells were then inoculated 1:33 into low sulfate medium with 0.5% methanol and 20 µCi/mL [^{35}S]methionine/cysteine and incubated until stationary growth phase was reached. At this time, methanol-induced exponential and stationary cultures and glucose adapting cultures were chased with unlabeled methionine and cysteine at final concentrations of 0.1 M each. At various times of chase, cell extracts were prepared and the radioactivity present in DHAS and AOX determined as described above. The data ([^{35}S] cpm/g cellular protein) was expressed relative to non-glucose adapting stationary cultures.

The ß-subunit of mitochondrial F_1 ATPase (F1ß) was immunoprecipitated from samples of the extracts of glucose repressed cells using modifications of the methods of Aris and Blobel (1989). Aliquots of *Staphylococcus aureus* (10-15% suspension of heat-killed, glutaraldehyde fixed cells; SA) were washed 3 times in 2 volumes of immunoprecipitation buffer (20 mM Tris/Cl, pH 7.8, 150 mM NaCl, 2 mM EDTA, 0.02% NaN_3; IP) + 0.5%BSA + 1%Tx100 + 0.2%SDS, then resuspended in 1 volume of the same buffer. Cell-free extracts were transferred to a tubes containing one tenth volume of washed and blocked SA

then incubated 1-2 h, 4°C to pre-clear the extracts then harvested by centrifugation for 10 min in a microfuge at full speed. Supernatants were transferred to fresh tubes and diluted 10-fold in IP + 0.5%BSA + 1% TX100 + 0.2% SDS to solubilize the proteins. Polyclonal antisera raised against F1β (Lewin and Norman, 1983) were added in amounts sufficient to bind to all available antigen and incubated overnight at 4°C. Washed, blocked SA was added at a proportion of 5 volumes of SA suspension/volume of antiserum used, incubated 1-2 h, 4°C, and the SA was pelleted in a microfuge at full speed for 3 min and the supernatant discarded. Precipitated immune complexes were washed in IP + 1% TX100 + 0.2% SDS and in IP alone. The antigens were eluted by adding buffer containing 10 mM Tris/Cl, pH 8.0 + 1 mM EDTA + 2% SDS + 1% ß mercaptoethanol + 20% sucrose to the samples, using the same volume of buffer as the original volume of SA suspension used. Samples were heated to 95°C for 2 min to aid dissociation of immune complexes. SA was removed by centrifugation (5 min) and the supernatant saved for polyacrylamide gel analysis. The immunoprecipitates were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose or dried and quantitated by phosphorimager analysis (Molecular Dynamics Phosphorimager).

Measurement of Overall Protein Degradation

Histidine auxotrophic yeast strains were precultured in YPD then inoculated 1:30 into medium containing 6.7 g/L yeast nitrogen base without amino acids (Difco), 2% glucose, 40mg/L histidine, 40 μg/L biotin, and 1 μCi/mL

[^{14}C]valine (Amersham). Cultures were incubated ~18h then washed twice in 6.7 g/L yeast nitrogen base without amino acids. Individual cultures were then divided into two equal portions and incubated with 10mM cold valine chase in the absence or presence of histidine (40mg/ml) in yeast nitrogen base without amino acids, 2% glucose and 40 µg/L biotin. Aliquots were collected on ice at 0, 5, and 8 hours of chase with the immediate addition of ice cold trichloroacetic acid to a final concentration of 20% w/v. The samples were incubated on ice for at least 1h then centrifuged. The supernatant was aspirated and kept separate from the pellet which was solubilized in 0.5mL Scintigest (Fisher Scientific Co.) The radioactivity in the supernatant (acid-soluble counts) and solubilized pellet (acid-insoluble counts) was counted on a Beckman LS5000TD scintillation counter. Percentage degradation was calculated as the ratio of TCA-soluble counts at each time point to acid-insoluble (protein-associated) counts at 0 hour of chase multiplied by 100 and normalized to one hour of chase.

Isolation of Glucose-Induced Selective Autophagy-Deficient (*gsa*) Mutants

Mutagenesis and Screening

Pichia pastoris cells (GS115) were mutagenized according to the methods of Cregg (1990). Briefly, single colonies were grown in YPD medium overnight to an A_{600} of 0.5 to 1.0 and then washed twice with 0.1M sodium citrate, pH 5.5. Cells were resuspended in 40 mL sodium citrate plus 100 µg/mL N-methyl-N'-nitro-N-nitrosoguanidine and incubated at room temperature

without shaking for 1 hour followed by detoxification by mixing with an equal volume of 5% sodium thiosulfate. Cells were then washed three times with sterile water and inoculated into 140 mL YPD cultures and incubated for 2 to 6 hours at 30°C. The mutagenized yeast were harvested and concentrated in fresh YPD to 10 A_{600} units and sterile glycerol was added to a final concentration of 30%, aliquoted, and allowed to equilibrate at room temperature for 2 hours and then frozen at -80°C for later use. Cells were viable for screening for > 1 year of storage.

Mutagenized cells were screened for the loss of the ability to degrade alcohol oxidase in response to a shift in carbon and energy source from methanol to glucose. The screening protocols are modifications of the direct colony assay procedures of Gleeson et al. (1984) and Tomlinson and Esser (1992). Aliquots of mutagenized yeast were thawed and diluted in water and sonicated for 30 s to break up cell clumps without reducing their viability. One hundred μL of the diluted cells were spread on MIM plates and incubated until colonies formed (5 to 6 days). These master plates were replicated to GA plates by placing sterile 85 mm nitrocellulose circles directly onto the master plates until they were soaked through and then placing onto GA plates. These plates were incubated at 30°C for 12 to 14 h during which time peroxisomes would be repressed in cells with a normal selective autophagy pathway. It was then necessary to spheroplast the cells to allow access to cellular contents for detection of peroxisomal AOX. To accomplish this, the

colony circles were treated as follows: the nitrocellulose circles are placed on paper filters soaked with 20 mM dithiothreitol in 67 mM potassium phosphate buffer, pH 7.5, containing 20 mM EDTA and incubated for 5 min at room temperature. The nitrocellulose circles were then moved to fresh paper filters soaked in 0.25 mg/mL Zymolyase 20T in 67 mM potassium phosphate buffer, pH 7.5 and incubated at 30°C for 1 hour. For colorimetric assay, the nitrocellulose circles were placed on fresh filters soaked in 0.13% methanol + 3.4 U/mL HRP + 0.56 mg/mL ABTS in 33 mM potassium phosphate, pH 7.5 and incubated at room temperature until color develops. The originals of the positive replica colonies were picked from the master plates and grown in YPD and mutant status was verified as follows: putative mutants were grown in MIM to stationary phase and the cultures were sampled at 0 h and 6 hours after addition of solid glucose to 2% final concentration and assaying for AOX and FDH activity. Measurement of the decrease in methanol-induced enzyme activity after glucose treatment allowed quantitation of the defect. The amount of activity present at the end of 6 h glucose treatment was compared to enzyme activity in the parental strain (see Fig 4-2).

Genetic Analysis

Backcrossing. In order to obtain mutant strains in which the defective gene of interest is the only mutated gene different from that found in the parental strain, the haploid mutants were sequentially mated to essentially wild type strains (GS115 or GS190-3) containing complementary auxotrophic markers and

haploid progeny recovered. Several rounds of this "backcrossing" procedure result in strains in which the only difference from the parental strain is the mutant gene of interest. A single round of backcrossing was accomplished as follows: the mutant strains were streaked on YPD plates in 2 cm X 2 cm patches and the parental strain of the complementary auxotrophy (i.e., His^- x Arg^-) was spread on YPD plates to form a lawn at a density of $1x10^7$ cells/plate and both were incubated at 30°C overnight. The lawn and the patch plates were both replica plated to a single SM plate and incubated overnight at 30°C to induce mating. These plates were then replicated to DSM plates (lacking amino acids) and incubated for 2 to 3 d, until diploid (prototrophic) colonies appeared. Colonies from these plates were streaked onto fresh DSM plates and incubated 1 to 2 d and then streaked to YPD plates overnight. Cells from this plate were streaked to SM plates and incubated 4 d at 30°C to induce meiosis and sporulation at which time some of these cells were harvested with an inoculation loop and etherized to kill any remaining diploid cells. Etherization consists of putting a loopful of cells from the SM plate into 1 mL sterile water, adding 1 mL diethylether, vortexing and incubating at room temperature for 20 min. An aliquot from this mixture was diluted 1:100 and 100 µL spread on YPD plates which were incubated for 2 d so that the spores germinate to grow vegetatively. Sterile water was placed on these plates and swirled to loosen some of the cells and collected; this cell suspension was diluted and plated on MIM to yield 100 to 500 colonies/plate. The resulting colonies were screened by direct colony assay

and then verified for the *gsa* phenotype as described above. Only *gsa* mutants of the opposite auxotrophy were selected, i.e., if the mutant strain was His^- before backcrossing, only Arg^- mutants were selected to ensure that the original mutant had not come through the procedure without backcrossing.

Complementation analysis. To determine the number of different mutant genes which were present in my collection of *gsa* mutants, the ability of diploids produced by mating different mutant strains to degrade peroxisomes was assessed. If the diploids produced by mating two mutant strains is normal then the strains are said to complement, indicating that the genes mutated in the two strains are different. Accordingly, if they do not complement, the two strains contain presumably different mutant alleles of the same gene. To accomplish this, a His^- backcross of one mutant strain was mated to an Arg^- mutant strain as described above, diploids were recovered from DSM plates and inoculated into YPD media and cultured until stationary. The ability to degrade peroxisomes was assessed as described above.

CHAPTER 3
GLUCOSE-INDUCED SELECTIVE AUTOPHAGY IN METHYLOTROPHIC YEASTS

Introduction

Certain species of yeast in several genera can grow in media containing methanol as the sole carbon and energy source, e.g., *Candida boidinii*, *Hansenula polymorpha*, and *Pichia pastoris*. At the time that *H. polymorpha* and *P. pastoris* are fully methanol-induced, i.e., grown up in methanolic media until the carbon source is depleted and thereby constituting a non-growing or stationary culture, the peroxisomal enzymes alcohol oxidase (AOX) and dihydroxyacetone synthase (DHAS) comprise ≥50% of the cells' total protein mass. These two proteins are important mediators of methanol utilization (see Fig. 1-1) and are virtually undetectable in cells grown with glucose as the sole carbon source (see Fig 3-1; Gleeson and Sudberry, 1988; Gould et al., 1992; Veenhuis et al., 1983). This being the case, it seems reasonable that as a cell adapts to new carbon sources it would be advantageous for the cell to rid itself of these proteins and to reutilize their component amino acids. In this study, I have chosen the ascomycetous methylotrophic yeasts *H. polymorpha* and *P. pastoris* to investigate the fate of enzymes necessary for the utilization of methanol when the carbon and energy source is changed. One of the initial

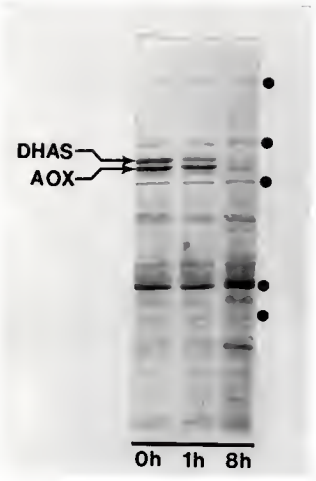

Figure 3-1. Comparison of the protein profiles of methanol-induced *P. pastoris* and cells undergoing glucose adaptation. Cultures of *P. pastoris* were grown to stationary phase in methanol then adapted to glucose for 8 h. Samples of equal volume were removed from cultures at 0, 1, and 8 h after the addition of glucose. Cell extracts were prepared, solubilized in SDS, and component proteins separated by SDS-PAGE and identified by Coomassie staining. Alcohol oxidase (AOX) at 74 kDa and dihydroxyacetone synthase (DHAS) at 78 kDa are identified (arrows). Five unknown proteins, the levels of which appear to remain relatively constant during glucose treatment, are identified by closed circles.

aims of the present study was to determine if the peroxisomal proteins were in fact degraded and by what mechanism. Autophagy has been most extensively described in mammalian cells, and in particular, rat liver cells (Ahlberg et al., 1985; Dunn 1990a,b; Kopitz et al., 1990; Mortimore et al., 1988). Experimentation conducted in these cells has on the whole shown autophagy, especially that induced by nutritional stress, to be a nonspecific process, i.e., soluble and organellar cytoplasmic components are taken up into autophagosomes or lysosomes without regard for their identities or their senescence. Similarly, in *S. cerevisiae* cells in which autophagy has been induced by nutritional stress, uptake of cytoplasmic components into the vacuole appears to be nonselective based on the fact that the contents of the vacuole in vacuolar protease mutant cells was indistinguishable from the cytoplasm itself (Takeshige et al., 1992; Baba et al., 1994). These results are not surprising considering that starvation-induced autophagy produces substrates for synthesis of replacement housekeeping proteins required to allow the cells to remain viable during poor nutritional conditions.

In the case of adaptation to glucose by cells previously adapted to methanol, in which the cells are undergoing major, rapid changes in the cellular content of specific enzymes in order to adapt to a change in carbon and energy source, the autophagic response might be expected to be more selective. In this case it may be hypothesized that a more limited subset of cellular proteins is being degraded and a different set of proteins synthesized. Rather than

degrade cytoplasmic proteins randomly, which in this event would seem to be not necessary and therefore energetically wasteful, specific proteins and organelles required to utilize methanol may be replaced by other specific proteins required to utilize glucose.

While several authors have investigated the disappearance of methanol-induced peroxisomes in methylotrophic yeasts (Bormann and Sahm, 1978; Bruinenberg et al., 1982; Hill et al., 1985; Veenhuis et al., 1978, 1983), previous studies have not directly measured protein degradation. Consequently, it has not been determined whether the decrease in morphologically identifiable peroxisomes and associated enzymes observed in response to the nutrient adaptation effect is due to selective or enhanced degradation or, rather, to a decline in enzyme/peroxisome synthesis in conjunction with unaltered, constitutive degradation.

The aims of this part of my study were as follows: 1) to determine whether the degradation of methanol-induced peroxisomes is brought about by autophagy; 2) to investigate the means by which methanol-induced peroxisomes are sequestered and the cellular site of degradation; and 3) to determine whether the degradation of these components is selectively enhanced during adaptation to glucose.

Loss of Methanol-Induced Enzymes During Glucose Adaptation

Degradation of Methanol Assimilating Enzymes

Several investigators have measured the loss of enzyme activity and protein in methanol-induced yeasts in various stages of growth (Bormann and Sahm, 1978; Bruinenberg et al., 1982; Hill et al., 1985; Veenhuis et al., 1978,1983). The present work is the first to measure synthesis and degradation in cells rapidly growing in methanol or grown to stationary phase in methanol. Degradation cannot be assumed to be equal to the loss in enzyme activity or protein because the cellular content of a given protein is the combined result of degradation and synthesis. By metabolically labeling cells during growth in methanol and adapting to glucose in chase media, I was able to unequivocally measure methanol-induced enzyme degradation. In addition, I estimated enzyme synthesis by quantifying incorporation of ^{35}S-labeled amino acids into peroxisomal enzyme proteins during logarithmic and stationary growth in methanol media.

The peroxisomal enzymes alcohol oxidase (AOX) and dihydroxyacetone synthase are very prevalent in methanol-induced *H. polymorpha* and *P. pastoris* such that the bands containing these proteins can be easily recognized by Coomassie staining gels in which cell-free extracts have been electrophoresed (see Fig 3-1; Douma et al., 1985). To measure degradation of peroxisomal proteins directly *P. pastoris* and *H. polymorpha* cells were metabolically labeled with [^{35}S]methionine/cysteine during the entire time cells

were growing in methanolic media containing low sulfate to stationary phase. At this point the cells were harvested and resuspended in media containing excess, unlabeled methionine and cysteine (to chase the radiolabeled amino acids) and glucose or methanol. Samples were taken at 0 and 3 hours of chase, cell-free extracts prepared and separated on 7.5% SDS-polyacrylamide gels. The bands corresponding to DHAS and AOX (see Fig. 3-1) were excised from the gels, solubilized and the radioactivity quantified in a scintillation counter. In some cases the gels were transferred to nitrocellulose blots to which film was exposed. In these cases the bands corresponding to AOX and DHAS on the resulting autoradiographs were quantified by laser densitometry. These data yielded essentially the same results as those in which bands were excised from the gels and counted. The results from both of these procedures were combined and are shown in Fig. 3-2. It can be observed that the peroxisomal proteins are stable in methanol but rapidly degraded in the presence of glucose. This indicates that the degradation of peroxisomal proteins is enhanced when the carbon and energy source is changed to glucose in both methylotrophic yeasts examined.

I next examined whether, since AOX and DHAS are stable in stationary cultures in methanolic media, synthesis of these proteins is low. In order to test this hypothesis the incorporation of radiolabeled methionine and cysteine into peroxisomal proteins was evaluated. *H. polymorpha* and *P. pastoris* cells were grown in low sulfate media containing methanol either to log or stationary growth phase. At this time cultures were either harvested and resuspended in glucose-containing media (stationary cultures) or maintained in methanolic media

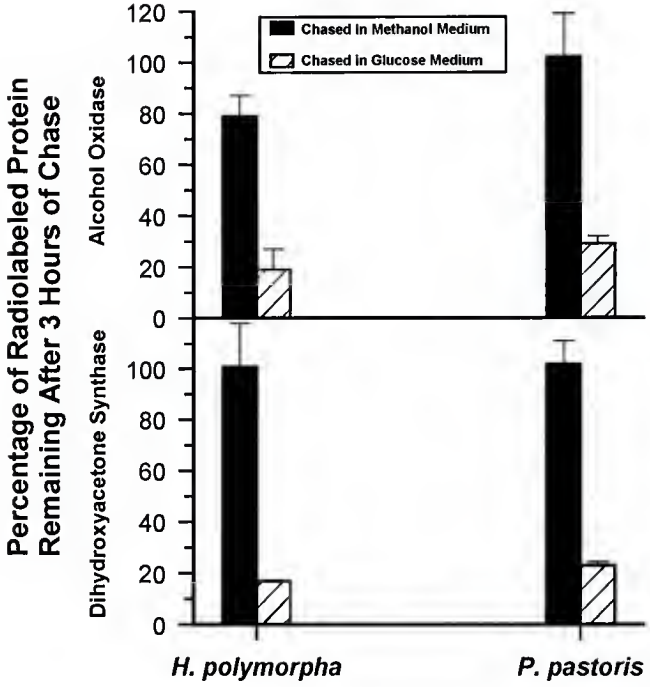

Figure 3-2. Turnover of peroxisomal proteins in methanol induction and glucose adaptation media. Cultures of *H. polymorpha* and *P. pastoris* were grown to stationary phase in low sulfur media with methanol as the sole carbon source, in the presence of [^{35}S]cysteine/methionine. At 0 h, unlabeled cysteine and methionine were added to chase the label in the absence (solid bars) or presence (hatched bars) of 2.0% glucose. Replicate samples were removed at 0 and 3 h of chase, cell-free extracts prepared and fractionated by SDS-PAGE. Radioactivity corresponding to dihydroxyacetone synthase and alcohol oxidase was quantified (see Chapter 2) and the values shown are the mean + s.e.m. for 3 or 4 determinations in two separate experiments.

(stationary and exponentially growing cultures). [^{35}S]methionine/cysteine was added to replicates of stationary cultures for 15 minutes every 2 hours during the first 8 hours after glucose addition. Exponential cultures were pulsed for 15 minutes. After the pulses cultures were harvested and cell-free extracts prepared and separated by SDS-PAGE and the bands corresponding to AOX and DHAS quantitated as above. These data show that peroxisomal enzyme synthesis is lowered by 95% during stationary phase relative to that during exponential growth (Fig. 3-3). Moreover, synthesis of these proteins is not significantly decreased after the addition of glucose.

In summary, these results verify that methanol-induced peroxisomal enzyme degradation is enhanced during adaptation to glucose. Furthermore, since peroxisomal enzyme synthesis is very low during stationary phase, any decrease in enzyme protein must be due to degradation under these growth conditions.

Further confirmation of the actual loss of proteins that function in the methanol assimilation pathway during glucose adaptation has been derived by use of immunoblots employing polyclonal antisera specific for the methanol-induced enzymes AOX (peroxisomal) and formate dehydrogenase (FDH; cytosolic). Cell-free extracts were prepared from hourly samples of *P. pastoris* cultures during 6 hours of glucose adaptation and were either assayed for AOX and FDH activity or the proteins separated on 7.5% SDS polyacrylamide gels prior to transfer to nitrocellulose. Immunoblot analysis was conducted according

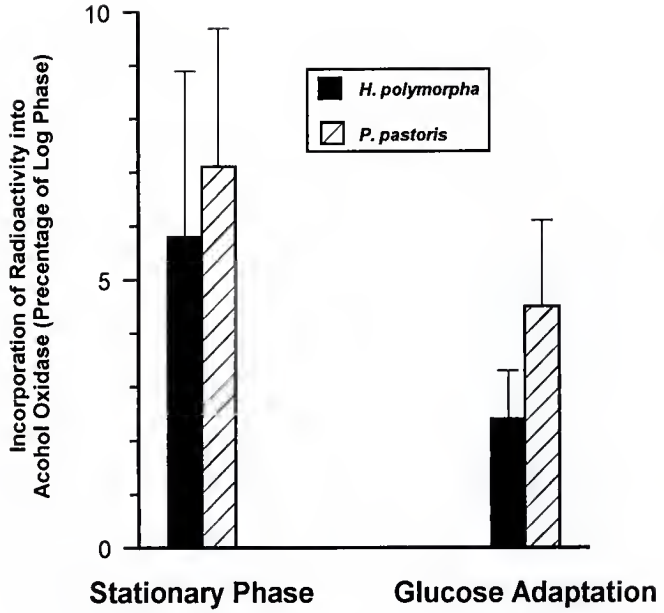

Figure 3-3. Synthesis of alcohol oxidase by *H. polymorpha* and *P. pastoris* during stationary phase in methanol-induced cultures and in glucose adapting cultures relative to synthesis during exponential growth (log phase). *H. polymorpha* and *P. pastoris* cultures were grown to exponential or stationary phase in low sulfur media containing methanol as the sole carbon and energy source. The incorporation of [^{35}S]cysteine/ methionine into alcohol oxidase during exponential phase, and at various points during the first 8 hours of stationary phase and glucose adaptation was quantitated as described in Chapter 2. The results represent the mean + s.e.m. of four determinations from two separate experiments and are expressed as a percentage of the incorporation of radioactivity into alcohol oxidase in methanol-induced exponentially growing cultures.

to the ECL protocol of Amersham utilizing polyclonal antibodies monospecific for either FDH or AOX at concentrations of 1:10,000.

The effects of glucose adaptation are presented in Fig 3-4 and clearly demonstrate the glucose-induced decrease in enzyme activity and protein in the case of both peroxisomal (AOX) and cytosolic (FDH) methanol catabolism enzymes.

Loss of Enzyme Activity

In order to rapidly measure degradation of methanol-induced enzymes during adaptation glucose, enzyme assays were conducted on samples taken from cultures adapting to these carbon sources at various time points. Cases in which measuring enzyme activity during adaptation is a reliable method for assessing enzyme protein degradation are those in which both the enzyme is being synthesized at a very low rate or not at all and in which inactivation is brought about by degradation. This is the circumstance for peroxisomes and cytosolic FDH during glucose adaptation. Furthermore, inasmuch as the objective of the present project is to investigate the manner in which peroxisomes are sequestered, rather than the act of degradation per se, if peroxisomal sequestration parallels enzyme inactivation then enzymes assays will be useful to assess the presence of these two proteins. Samples were taken and assayed by volume rather than by protein concentration on the basis of enzyme activity per methanol-induced cell volume at 0 hour of glucose adaptation. The assumption is that in any volumetric sample taken during the

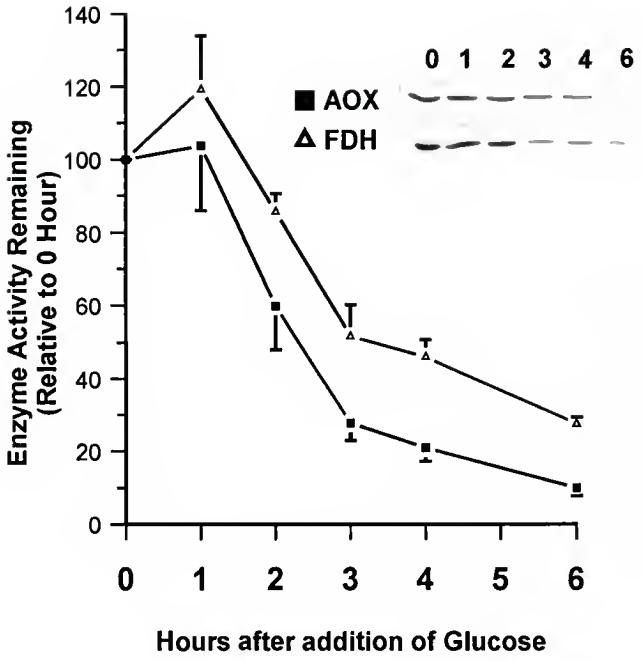

Figure 3-4. Degradation of peroxisomal and cytosolic enzymes during glucose adaptation. *Pichia pastoris* cells (GS115) were cultured in methanol induction medium until stationary at which time glucose was added to begin adaptation. Cell-free extracts were prepared at selected times and peroxisomal alcohol oxidase (AOX) and cytosolic formate dehydrogenase (FDH) activities measured. The values represent the average ± s.e.m. of six determinations. Protein levels of AOX and FDH within extracts of glucose adapting cells were analyzed by immunoblots (inset).

course of adaptation, the same number of cells which were present in a methanol-induced state at 0 h will be also present in that sample.

Autophagy of Peroxisomes Induced By Glucose

Ultrastructural Examination of Autophagy

P. pastoris and *H. polymorpha* cells were fixed for transmission electron microscopy utilizing a potassium permanganate protocol which is useful for detecting membrane events (Veenhuis et al., 1983; Tuttle et al., 1993). When grown to stationary phase in media originally containing 0.5% methanol as the sole carbon and energy source, the yeast cells contain many large peroxisomes (Fig 3-5B; Veenhuis et al., 1983; Gould et al., 1992). The peroxisomes are observed assembled in clusters in the cytoplasm, often adjacent to endoplasmic reticulum, rounded (i.e., spherical or lobulated) vacuoles, and other cytoplasmic organelles. In contrast, cells grown in glucose-containing media, i.e., not previously induced in methanol, are devoid of identifiable peroxisomes (Fig. 3-5A; Veenhuis et al., 1983; Gould et al., 1992). The objective of these ultrastructural studies, then was to investigate how the cells were able to dispose of the methanol-induced peroxisomes and return to the morphology typical of glucose-grown cells. In methanol-induced *P. pastoris* drastic changes in the ultrastructure of the vacuole are observed within 0.5 to 1 hour after the addition of glucose (Fig 3-6). The earliest change noted is the contortion of the vacuole to a cup-shaped appearance with a cluster of peroxisomes in the hollow formed (Fig 3-6A,B).

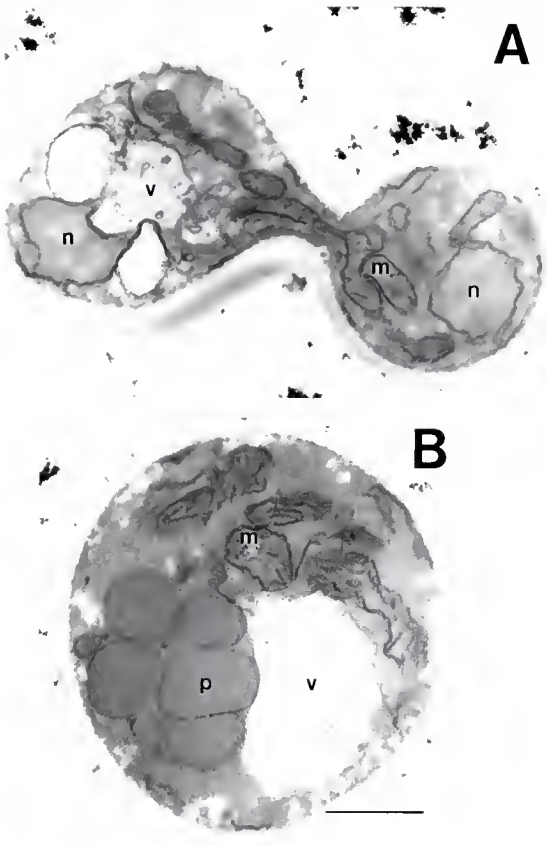

Figure 3-5. Ultrastructure of methylotrophic yeasts grown in glucose and methanol. *H. polymorpha* (A) and *P. pastoris* (B) cells were incubated in cultures containing glucose (A) or methanol (B) as the sole carbon and energy sources then fixed with potassium permanganate and examined by transmission electron microscopy. p = peroxisome; m = mitochondrion; n = nucleus; v = vacuole; Bar = 0.5 μm.

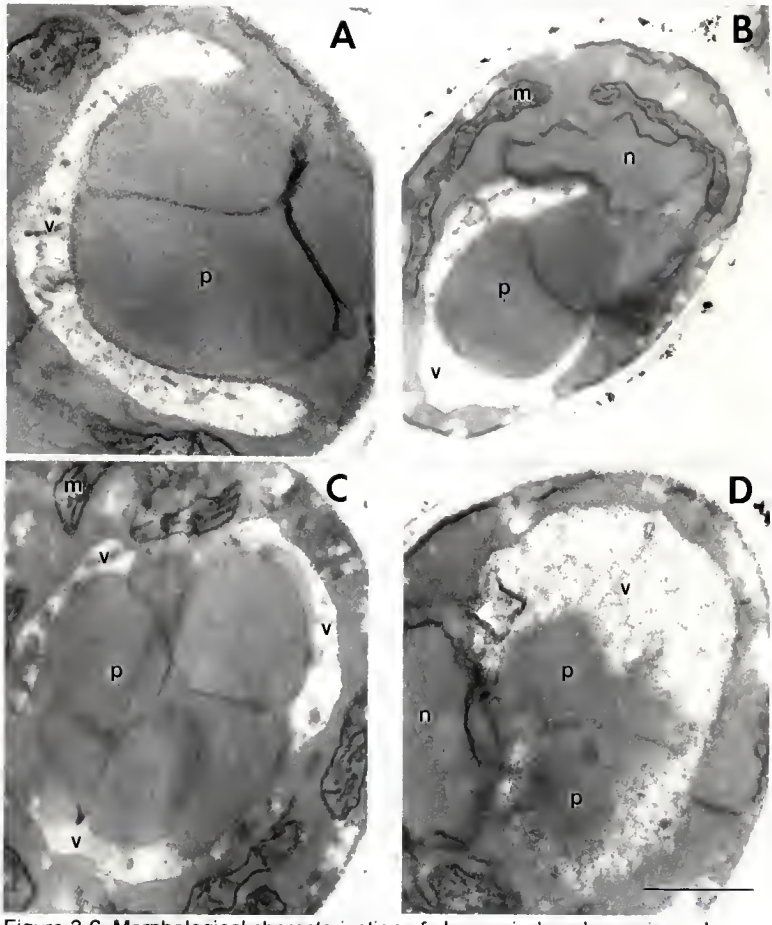

Figure 3-6. Morphological characterization of glucose-induced peroxisomal degradation in *P. pastoris*. *P. pastoris* was grown in methanol medium until stationary growth was achieved. Glucose was then added to the cultures to a final concentration of 2.0% then incubated for one additional hour and potassium permanganate fixed as described in Chapter 2. p= peroxisome; v = vacuole; m = mitochondrion; n = nucleus; Bar = 0.5 μm.

In other micrographs, vacuoles are seen completely encircling a peroxisome cluster (Fig. 3-6C). Another prevalent configuration observed is a cluster of peroxisomes entirely within the vacuolar lumen with the members of a peroxisome cluster observed in various stages of degradation (Fig 3-6D).

These observations suggest a course of events reminiscent of mammalian microautophagy (see Chapter 1). In some way the vacuole is triggered to change shape, distending to cup around a cluster of peroxisomes and continuing to extend arms until the peroxisomes are completely surrounded but still not taken up into the vacuole (Fig 3-6A-C). Subsequently, the inner vacuole membrane disappears, perhaps by degradation or fusion with the peroxisome membranes, and the peroxisomes are thereby incorporated into the vacuolar lumen were they are degraded by the vacuole-resident hydrolases (Fig 3-6D).

The ultrastructure of *H. polymorpha* cells during glucose adaptation is strikingly different. In this yeast, during the first hour of glucose treatment, individual peroxisomes within a cluster can be seen surrounded by from 2 to 12 extra membrane layers of unknown origin (Fig. 3-7B; Veenhuis et al., 1983). In some cells peroxisomes are seen singly or in small groups in the lumen of the vacuole, sometimes apparently partially degraded (Fig. 3-7B). A possible sequence of events has been described by Veenhuis et al. (1983) and bears a marked resemblance to mammalian macroautophagy in which cytoplasmic components are first sequestered by a double membrane body forming an autophagosome (Dunn, 1990a) which fuses with lysosomes forming a

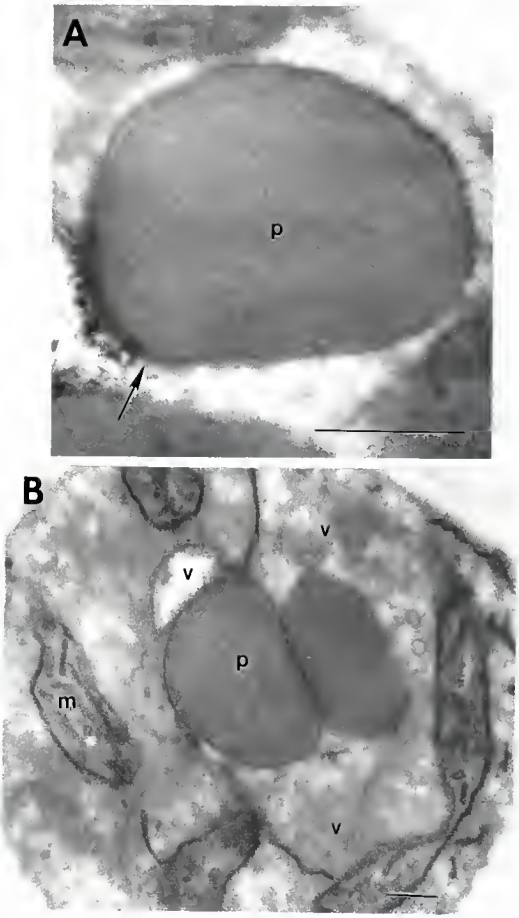

Figure 3-7. Morphological characterization of glucose-induced peroxisomal degradation in *H. polymorpha*. *H. polymorpha* was grown in methanol medium until stationary growth was achieved. Glucose was then added to the cultures to a final concentration of 2.0% then incubated for one additional hour and potassium permanganate fixed as described in Chapter 1. Arrow in A points to outer autophagosome membrane layer. p = peroxisome; v = vacuole; m = mitochondrion; Bar = 0.5 µm.

degradative autolysosome (Dunn, 1990b). A similar course of events may take place in the case of *H. polymorpha*. One posssible scenario is that membranes of unknown origin (possibly endoplasmic reticulum cisternae, if true to the mammalian process) sequester individual peroxisomes from the remainder of the cytosol forming an autophagosome. The limiting membranes of the autophagosome may possess the ability to recognize and fuse with the degradative vacuole whereby peroxisomes are deposited into the vacuolar lumen and degraded.

Vacuolar Degradation-Deficient Mutants Verify the Site of Peroxisome Degradation During Glucose Adaptation

The biogenesis of the vacuole is presently being studied in *Saccharomyces cerevisiae* (see Klionsky et al., 1990 for review) and it has been determined that the vacuolar endoproteases proteinase A (PrA) and proteinase B (PrB) are responsible for self-activating their zymogen forms as well as for the zymogen forms of several other major vacuolar proteases such as carboxypeptidase Y (CPY; see Chapter 1). In the absence of functional PrA and PrB, the proteolytic capacity of the vacuole is sharply curtailed. The homologs of PrA and PrB have been identified in *P. pastoris* and strains prepared by homologous recombination which lack functional forms of either or both of these proteases (M.A.G. Gleeson, personal communication). Initially these strains were tested to determine what effect these gene knockouts had on vacuolar function.

Analysis of PrA/PrB-Defective Strains

Enzyme activity assays were conducted for the presence of active PrA and CPY according to Jones (1977; 1991b). In SMD1163 (PrA^-/PrB^-) and SMD 1168 (PrA^-), PrA activity was decreased by ~80% compared to the wild type strain GS115. CPY activity was evaluated utilizing a semi-qualitative colorimetric colony assay, and indicated minimal CPY activity in both PrA^- strains. Interestingly, a color developed in SMD1165 (PrB^-) intermediate in intensity to that of the wild type strain and the PrA^- strains suggesting a more stringent requirement for PrA in zymogen activation than for PrB, consistent with the *S. cerevisiae* literature (Klionsky et al., 1990).

It has been shown in *S. cerevisiae* that culturing cells in media lacking nitrogen, carbon, or essential amino acids causes a large increase in nonspecific autophagic sequestration of cytoplasmic components and their degradation in the vacuole (Teichert et al., 1989; Takeshige et al., 1992; Baba et al., 1994). In *S. cerevisiae* mutants lacking PrA and PrB the increase in overall protein degradation rate due to nutrient deprivation is abolished. *P. pastoris* strains with and without functional PrA and/or PrB were tested for their ability to degrade endogenous proteins in response to amino acid deprivation. Cells were metabolically labeled in low sulfate containing media containing [^{35}S] methionine and cysteine (see Chapter 2) and glucose until stationary then harvested, washed and resuspended in chase media containing or lacking histidine (an amino acid required for growth of these strains). Overall protein degradation

was estimated by quantifying the ratio of trichloroacetic acid-soluble and insoluble counts in serial samples (Fig 3-8). The results indicate that in the strain containing functional copies of the genes encoding PrA and PrB (GS115) depriving the cells of histidine caused an increase in overall protein degradation (compare open squares to closed squares in Fig. 3-8). This indicates that during this type of nutritional stress the cell responds by increasing the degradation of cellular proteins. On the contrary, depriving PrA and/or PrB-defective strains of histidine caused no increase in protein degradation, indicating that these proteases are essential for starvation-induced degradation.

These data suggest that the homologs of PrA and PrB in *P. pastoris* are essential for starvation-induced degradation. Furthermore, these proteases are implicated in the activation of vacuolar zymogens suggesting a role in vacuolar biogenesis similar to their counterparts in *S. cerevisiae* (see Chapter 1).

Peroxisome Degradation in PrA/PrB-Deficient Strains During Glucose Adaptation

Glucose adaptation experiments were performed on the PrA/PrB double knockout strain of *P. pastoris* (SMD1163). The cultures were grown to stationary phase in methanolic media then glucose was added to the cultures to turn on autophagy of peroxisomes; as in previous experiments with GS115, samples were taken at the time of glucose addition and 6 h later and cell-free extracts prepared. The extracts were assayed for alcohol oxidase and formate dehydrogenase (FDH) activity and the results are represented in Figure 3-9A. The degradation of AOX and FDH was almost completely blocked in these

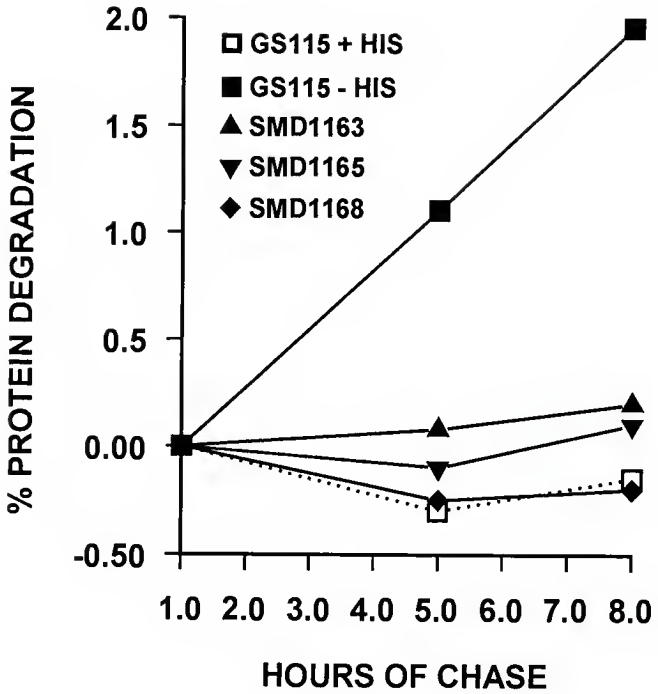

Figure 3-8. Protein degradation induced by histidine starvation is blocked in the absence of functional proteinases A and B. Parental *P. pastoris* (GS115: *his4*) cells were metabolically labeled with [^{14}C]valine and then chased with media containing cold valine in the absence or presence of histidine (HIS). Proteinase mutant strains, SMD1163 (*his4, pep4, prb1*); SMD1165 (*his4, prb1*); and SMD 1168 (*his4, pep4*), were labeled with [^{14}C]valine and chased in the absence of histidine. The release of trichloroacetic acid-soluble radioactivity was measured and the percent cellular protein degraded calculated as a percentage of total radioactivity at zero hour.

mutants. These data show that, in the absence of PrA and PrB, these cells are not able to degrade either AOX or FDH. Based on previous data that PrA and PrB are required for vacuolar degradation, this suggests that the vacuole is the site of degradation of both peroxisomes and the cytosolic enzyme FDH.

Ultrastructural Observations During Glucose Adaptation

The effect of glucose adaptation was also studied at the ultrastructural level in the putative vacuolar mutant strain of *P. pastoris* (SMD1163). Methanol-induced cells were sampled when grown to stationary phase (0 h) and at 6 h after the addition of glucose and fixed for morphological examination by the potassium permanganate procedure to enable visualization of membrane events (Fig. 3-9B). At 0 h, the mutant cells exhibited clusters of peroxisomes and the other cytoplasmic organelles with no apparent differences from the parental strain (not shown). After 6 h of glucose adaptation, the vacuoles of the putative vacuolar mutants were filled with undegraded peroxisomes (Fig. 3-9B). Other recognizable organelles were not observed in the vacuole. This result suggests that the vacuole is unhindered in its ability to sequester the peroxisomes but that it lacks the proteolytic functionality required to degrade them.

The data demonstrate that the *P. pastoris* homologs of PrA and PrB are required for vacuole-mediated degradation. Evidence is presented that suggests that these endoproteases act as vacuolar zymogen activators, a function they have been shown to serve in *S. cerevisiae*. Mutants of *P. pastoris* defective in PrA and PrB activity can be utilized to determine whether a

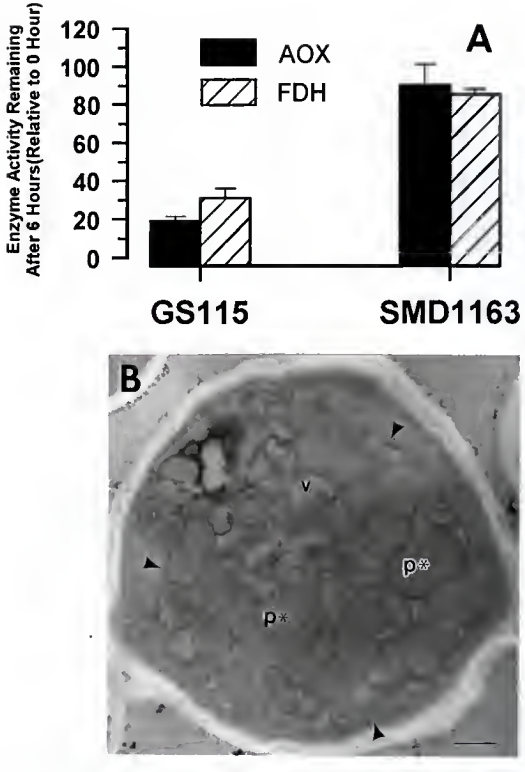

Figure 3-9. Proteinase mutants are not able to degrade methanol-induced components during glucose adaptation. Parental GS115 (*his*) and mutant SMD1163 (*his*, *pep4*, *prb1*) cells were grown in methanol then adapted to glucose. Alcohol oxidase (AOX) and formate dehydrogenase (FDH) activity were determined at 0 hours and 6 hours of glucose adaptation (A). The values represent the average + s.e.m. of at least three determinations done at 6 hours of adaptation and is reported as a percentage of that measured at zero hour. At 6 hours of glucose adaptation, the mutant cells were fixed and processed for ultrastructural examination (B). Intact peroxisomes (*) were observed within the vacuoles (v) of proteinase mutant cells (b). Arrowheads indicate the perimeter of the yeast vacuole. p = peroxisome; Bar: 0.5 μm.

particular degradation event takes place in the vacuole by evaluating biochemically whether or not a decrease in degradation has occurred in themutant strain relative to the wild type (see Fig. 3-8; 3-9B). Additionally, ultrastructural analysis revealed an accumulation of undegraded peroxisomes in the vacuole of protease mutants, suggesting the vacuole as the normal site of degradation (3-9B). By utilizing these mutants in this manner, the vacuole has been shown to be the normal site of degradation of methanol-induced peroxisomes and FDH during glucose adaptation (see Fig. 3-9). The vacuole also plays a major rôle in the degradation of cellular components during starvation for essential amino acids (see Fig. 3-8).

Selective Autophagy

To directly measure the degradation of mitochondrial proteins during glucose adaptation in *H. polymorpha* and *P. pastoris*, cells were metabolically labeled with [^{35}S]cysteine and methionine during growth to stationary phase in methanolic media. The cultures were then chased in the presence of glucose and the mitochondrial protein F_1-ATPase, β subunit (F1β) was immunoprecipitated from cell-free extracts generated at 0, 1, and 3 hours of chase (Tuttle et al., 1993). The amount of label associated with F1β was quantitated by phosphor-imaging after separating the extracts by 7.5% SDS-PAGE. The amount of label incorporated into the peroxisomal enzymes AOX and DHAS was also quantitated as above (see Fig. 3-2, 3-3). Figure 3-10 shows

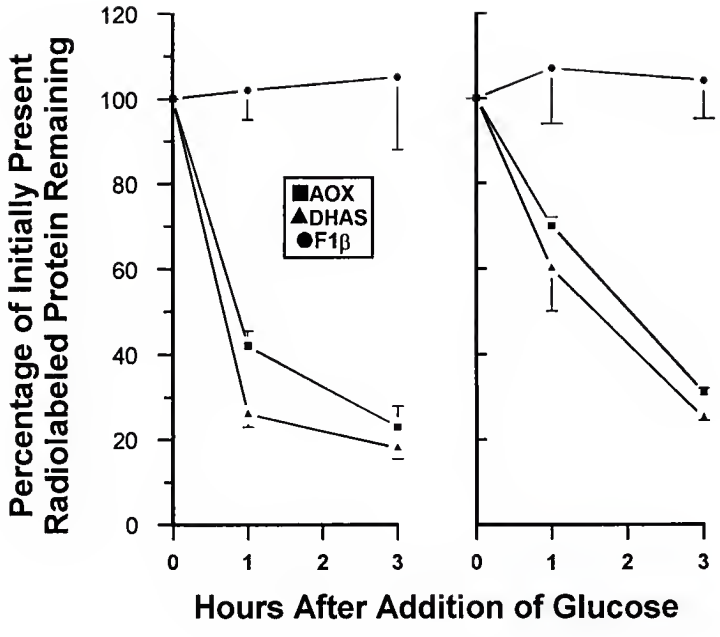

Figure 3-10. Degradation of peroxisomal and mitochondrial proteins during glucose-mediated peroxisome removal in *H. polymorpha* and *P. pastoris*. Cultures of yeast were grown to stationary phase in low sulfur media with methanol as the sole carbon source, in the presence of ^{35}S-methionine/cysteine. At 0 hour solid glucose was added to a final concentration of 2% to begin adaptation and solid methionine and cysteine were added to prevent reutilization (chase) of the radioisotope. Duplicate samples were removed at various times, protein extracts were prepared and analyzed as described in Chapter 2. For F_1 ATPase, ß-subunit (F1ß), protein was immunoprecipitated from the protein extracts, fractionated by electrophoresis and quantified with a Phosphorimager. AOX and DHAS were quantified by counting the radioactivity associated with the respective protein bands excised from Coomassie stained gels. Values shown are the mean ± SEM for 3 or 4 determinations in three separate experiments. AOX = alcohol oxidase; DHAS = dihydroxyacetone synthase.

the results of this analysis which indicate that while peroxisomal proteins are rapidly degraded, the mitochondrial protein F1β is stable.

Ultrastructural evidence also suggests that the autophagic degradation of peroxisomes during glucose adaptation is selective (see Fig. 3-6, 3-7, 3-9). In both *H. polymorpha* and *P. pastoris*, mitochondria or other cytoplasmic organelles were never seen sequestered along with peroxisomes.

Chapter Summary

Utilizing metabolic labeling/chase protocols in both *H. polymorpha* and *P. pastoris*, it was demonstrated that the synthesis of the peroxisomal enzymes DHAS and AOX was very low during stationary phase in methanol-induced cultures and therefore the disappearance of DHAS and AOX proteins which occurs during glucose adaptation must be due to enhanced degradation (see Fig. 3-3). It was also directly demonstrated that these peroxisomal proteins are quite stable in stationary methanolic cultures and their degradation is rapidly enhanced after the addition of glucose (see Fig. 3-2).

In cultures of *H. polymorpha* and *P. pastoris* grown to stationary phase in methanolic media, the peroxisomal enzymes AOX and DHAS are synthesized at low rates and are quite stable (see Fig. 3-2, 3-3). Upon addition of glucose to these cultures, the peroxisomal enzymes are rapidly degraded (Fig. 3-2, 3-4). In *P. pastoris*, clusters of peroxisomes are sequestered directly by the vacuole and degraded there as in microautophagy (see Fig. 3-6). In *H. polymorpha*,

individual peroxisomes are sequestered by multiple membrane layers and then fuse with the degradative yeast vacuole and deposit the peroxisomes into the vacuole where they are degraded as in macroautophagy (see Fig. 3-7; Veenhuis et al., 1983).

I have provided evidence that PrA and PrB are vacuolar proteases which act similarly to their counterparts in *S. cerevisiae*. These proteases are required for the proteolytic function of the vacuole, due to their innate endoproteolytic activity and probably by activating zymogen forms of vacuolar proteases (e.g., CPY, see above). This evidence consists of 1) the inhibition of degradation in histidine-starved vacuolar mutant strains (see Fig. 3-8); 2) the absence of CpY activity in cells lacking functional PrA and PrB; and 3) the accumulation of undegraded peroxisomes in the vacuole during glucose adaptation (see Fig. 3-9B. Utilizing these mutants, I have demonstrated that in *P. pastoris* the vacuole is the site of peroxisomal degradation during adaptation to glucose (see Fig. 3-9). In addition, the cytosolic methanol catabolic enzyme FDH is also degraded in the vacuole although the method of vacuolar uptake is unclear (see Fig. 3-9).

Finally, I presented evidence that peroxisome degradation induced by glucose is selective in that mitochondrial components that are not degraded simultaneously. This evidence is both biochemical (see Fig. 3-1; 3-10) and morphological (see Fig. 3-6, 3-7). All the evidence presented indicates that the degradation of peroxisomes occurs due to autophagy. Morphologic evidence

shows that peroxisomes are taken up into the vacuole as a whole where their constituent enzymes are degraded nonspecifically.

CHAPTER 4
ISOLATION AND CHARACTERIZATION OF *PICHIA PASTORIS* MUTANTS DEFECTIVE IN GLUCOSE-INDUCED SELECTIVE AUTOPHAGY

Introduction

An important reason that one uses ascomycetous yeasts to study eukaryotic systems is the ease of identification of genes that function in a pathway of interest afforded by certain of these yeasts. Since they can be maintained indefinitely in the haploid state, mutations have only to affect the single allele of a given gene to affect the phenotype. These mutations can be brought about by random mutagenesis or by gene-replacement with a cloned allele and identified by screening for a phenotype which suggests a defect in the pathway being studied. Subsequently, haploid mutant strains can be mated with other mutants to identify different complementation groups (affected genes) present in the mated mutants. Alternatively, haploid mutants can be mated to a strain with a wild type background (backcrossing) to produce diploids with a normal phenotype (if the mutation is recessive) which can then be stimulated to undergo meiosis, yielding haploid progeny, some mutant, some wild type for the gene of interest. During meiosis in yeast, homologous recombination events are common so that after several rounds of backcrossing, haploid strains may be

obtained in which the gene of interest will be the only mutated gene (aside from selectable auxotrophic markers, e.g., HIS4).

Genes may be cloned by transforming haploid mutants with a plasmid-borne library, assaying for complementation, and then recovering the DNA in rescued mutants. Sequence analysis leads to the ready identification of the proteins in the pathway of interest. Further genetic manipulation and study of the proteins can yield data on protein localization, interactions with other cellular components, structure, and function.

Much time and effort has been expended on the investigation of the regulation and mechanism of autophagy in mammalian systems (see Chapter 1). This work has revealed the existence of regulatory pathways which stimulate autophagy (e.g., starvation for certain amino acids) and some of the morphological features which led to the designation of macroautophagy and microautophagy. Unfortunately, due to the difficulty of performing classical and molecular genetic analyses in higher eukaryotes, the molecular mechanisms of these phenomena remain almost entirely unknown.

In order to take full advantage of the model for selective autophagy which the methylotrophic yeast system provides (see Chapter 3), I undertook to develop a system for the screening, verification, and characterization of *P. pastoris* strains which are defective in glucose-induced selective autophagy. The specific aims of this part of the present study were: 1) to chemically mutagenize an essentially wild type strain of *P. pastoris*; 2) screen for mutant

colonies which are unable to degrade methanol-induced peroxisomes in response to glucose; 3) verify and characterize the mutant phenotype; 4) and backcross the mutants and assign complementation groups.

Mutagenesis, Screening, and Verification

A strain of *P. pastoris* which is wild type except for the HIS4 selectable marker for histidine auxotrophy (GS115) was mutagenized using N-methyl-N'-nitro-N-nitrosoguanidine (which mainly causes base conversions leading to point mutations) according to the methods of Cregg et al. (1990). After treatment for 1 hour, leading to a 95% kill rate, cells were aliquoted and frozen at -80°C until tested for autophagy deficiency.

In preparation for screening, mutagenized cells were spread on MIM agar plates containing 0.5% methanol (master plates). The plates were incubated for 4 to 5 days to allow 1 to 2 mm colonies to form, at which time the plates were replicated onto nitrocellulose circles which were subsequently placed onto GA agar plates (containing 2.0% dextrose) or fresh MIM plates (positive control for the assay; see Fig. 4-1A) and incubated for 14 to 16 h. At this time the AOX-containing peroxisomes in normal cells will have degraded, therefore the strategy for screening was to identify colonies which still retained AOX activity after this period of adaptation to glucose. In order to do this, the replica-containing nitrocellulose circles were submitted to colorimetric direct colony assays for AOX activity as described in Chapter 2 and colonies retaining AOX activity (Fig. 4-1B) were selected from the master plates for verification and further characterization.

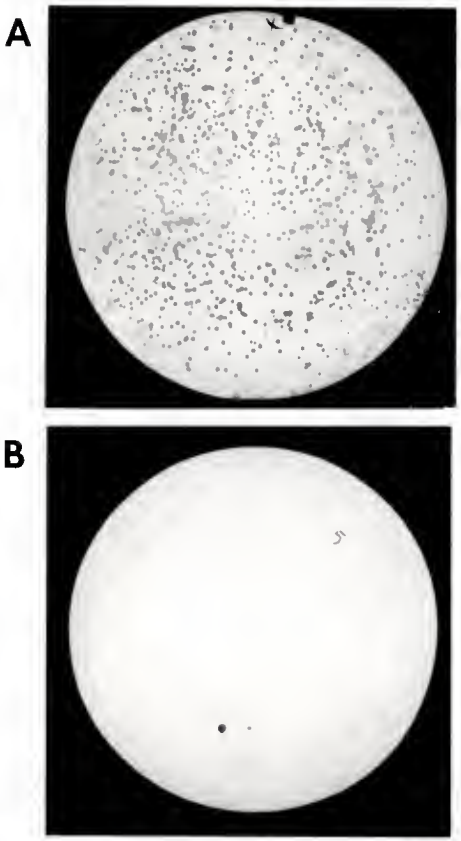

Figure 4-1. Screening for glucose-induced selective autophagy-deficient mutants (*gsa*) by direct colony assay for alcohol oxidase. Mutagenized colonies were incubated on MIM plates for 5 d then replicated to nitrocellulose circles. The nitrocellulose circles were then placed on fresh MIM plates (A; positive control) or glucose plates (B) and incubated 14 h to initiate glucose-mediated peroxisome degradation. The colonies in (A) all show positive reaction product while in (B) most of the colonies are not visible because they have degraded the methanol-induced peroxisomes and so remain white. Putative *gsa* mutants retain alcohol oxidase activity after glucose treatment (dark colonies in B).

In this way, 48 positive colonies were selected out of approximately 200,000 colonies screened. These 48 colonies underwent a verification procedure which consisted of the same procedure as described in Chapter 3 to quantitate the effect of glucose adaptation on methanol-induced proteins. Briefly, the colonies were grown up in YPD precultures, inoculated 1:30 into 20 mL MIM cultures, incubated ~2 d, then glucose was added to a final concentration of 2% to initiate glucose adaptation. Samples were taken at 0 and 6 h after addition of glucose and peroxisomal AOX and cytosolic FDH activity assayed to quantitate the putative autophagy defect during glucose adaptation.

Of these 48 positive colonies, 15 were verified to be defective in AOX and/or FDH degradation to the extent that they retained at least 2-fold more enzyme activity than the parental GS115 cells after 6 h glucose adaptation (Fig. 4-2). These 15 strains were tentatively designated glucose-induced selective autophagy-deficient (*gsa* mutants). Further confirmational assays indicated that the autophagy deficiency was variable from culture to culture for some of these strains, suggesting the putative strains were not clonal either 1) because of contamination with other strains due to the clumping characteristic of *P. pastoris* cells in culture or 2) because reversion of some of the mutant cells to the parental (or other) allele caused mixed populations in the cultures. Three of the original isolates, designated *gsa*4.3, *gsa*12.1, and *gsa*13.1 (see Fig. 4-2), were selected for backcrossing and complementation analysis by virtue of the severity of the exhibited autophagy defects and their relative consistency in tests of glucose adaptation.

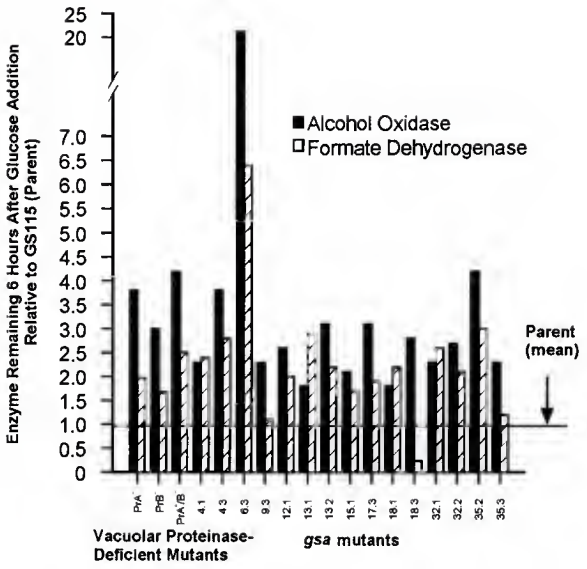

Figure 4-2. Comparison of degradation of methanol-induced enzymes in vacuolar protease-deficient mutants, *gsa* mutants, and the parental strain (GS115). The amount of alcohol oxidase and formate dehydrogenase activity present in samples removed from cultures 6 h after glucose addition relative to that present at 0 h was compared among vacuolar protease mutants lacking PrA and/or PrB, the 15 original isolates of the screening procedure for *gsa* mutants which were considered verified (designated by numerals on X axis; see text for details), and GS115. The results shown are normalized to GS115, the mean for which is set at 1.0 (horizontal line).

Backcrossing and Complementation

Backcrossing was carried out by replica plating the histidine-requiring *gsa* mutants with an arginine-requiring strain with a wild type background (GS190-3) to SM agar plates, lacking nitrogen, causing them to mate. Diploids were selected on plates lacking amino acids (DSM) since the auxotrophic mutations should complement to produce a prototrophic diploid. The diploids where then forced to undergo meiosis on the same nitrogen-free agar medium which causes them to mate (SM) and the resulting spores are regenerated to vegetative growth, then screened for the *gsa* phenotype by direct colony assay and for auxotrophy. Importantly, the *gsa* mutants selected from this backcross were arginine auxotrophs, ensuring that the resultant *gsa* backcross could not have gone through the procedure without mating and the attendant homologous recombination events which are the main purpose of backcrossing. In other words, the genotype of the *gsa* mutant selected from crossing of a *gsa/his4* strain with a GSA/*arg4* strain would be *gsa/arg4* (see Table 2-1).

In the next step in the backcrossing regimen, a *gsa/arg4* strain was crossed to *GSA*115 (GSA/*his4*) and the end product was a *gsa/his4* strain. In order to obtain a strain that reliably contains only a singly *gsa* mutation, 4 to 6 rounds of backcrossing are necessary.

Complementation analysis was carried out in the same manner, with the exception that cells of one histidine auxotrophic *gsa* clone were mated with cells of an arginine auxotrophic clone of a different *gsa* isolate. The resulting diploid

strains were tested for the ability to degrade methanol-induced AOX and FDH in response to glucose in the usual manner. Strains were considered complementary, i.e., contained mutations in different genes, if AOX was degraded at the wild type rate. Accordingly, strains which did not complement, degraded AOX at a rate significantly slower than the wild type, and were considered to contain different mutant alleles of the same gene. Controls for complementation analysis consisted of crossing *gsa* mutants to one of the wild type strains (positive control, diploid exhibits normal rate of AOX degradation; complementation of the *gsa* defect by the wild type strains also demonstrates the recessive nature of the mutant allele) and crossing a given *gsa* clone to a clone containing the same mutant allele, i.e., a backcrossed strain of the same clone, but a different auxotrophic marker (negative control in which diploid exhibits the mutant rate of AOX degradation).

This analysis has revealed two complementation groups designated *gsa1* and *gsa2* and two mutant alleles of *gsa2* have been isolated, designated *gsa2-1* and *gsa2-2*. The strains carrying these mutations have been named according to their *gsa* alleles: WDY1 (derived from original isolate *gsa*12.1), WDY2 (derived from *gsa*4.3), and WDY3 (derived from *gsa*13.1; see Table 2-1, Fig. 4-2).

Biochemical and Ultrastructural Examination of WDY1 and WDY2

After backcrossing WDY1 and WDY3 once and WDY2 four times, these strains were further analyzed for their ability to degrade methanol-induced peroxisomes and FDH in response to glucose (Fig. 4-3A). This was tested by the usual 6 h glucose adaptation procedures as described in Chapter 2. These three strains were all severely defective in the removal of both methanol-induced peroxisomes (AOX: solid bars in Fig. 4-3A; compare mutants to GS115) and cytosolic FDH (Fig. 4-3A, hatched).

Using the same cell-free extracts as for the AOX and FDH assays described in the preceding paragraph, these strains were tested to determine whether they had lost the selectivity of degradation exhibited by the parental strain (GS115; see Fig. 3-6, 3-12). This was accomplished by measuring enzyme activities not acutely regulated by glucose in wild type *P. pastoris*, i.e., mitochondrial cytochrome *c* oxidase (CCO) and cytosolic fructose-1,6-bisphosphatase (FBP; Fig. 4-3B). After 6 hours of glucose adaptation, the activities of these two enzymes remain relatively constant in parental GS115, suggesting that mitochondria and cytosolic components are not randomly degraded with the methanol-induced peroxisomes and FDH. Similarly, in the *gsa* mutants, the levels of these enzymes remain constant or 20 to 50% higher (Fig 4-3B). These data suggest that selectivity of degradation is not lost in these mutants since, while peroxisomes and FDH are degraded to some degree, the degradation of these components is not enhanced relative to the wild type. In

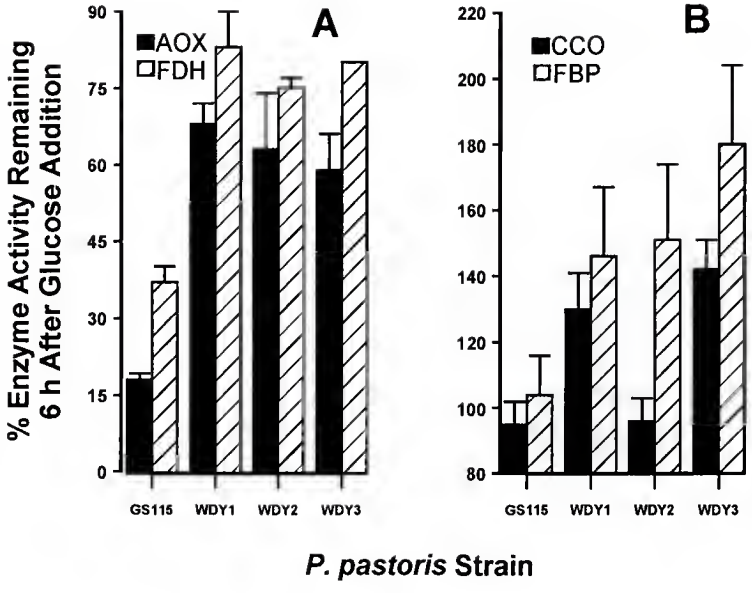

Figure 4-3. Analysis of specific and nonspecific degradation by *gsa* mutants. Enzyme assays were conducted on cell-free extracts prepared from various mutant and wild type (GS115) strains at 0 and 6 h of glucose adaptation. The results are expressed as the percentage of enzyme activity present at 6 h relative to that present at 0h. Activity of the glucose-sensitive proteins alcohol oxidase (AOX) and formate dehydrogenase (FDH; A). Activity of the glucose-insensitive proteins cytochrome *c* oxidase (CCO) and fructose-1,6-bisphosphatase (FBP; B). Bars represent mean + s.e.m.

fact, the levels of CCO and FBP tend to be higher in the *gsa* mutants, suggesting that a certain level of constitutive degradation of these cellular constituents occurring in stationary phase in methanol in the wild type cells is blocked in the mutants. These data suggest that constitutive degradation of cytoplasmic components also proceeds by autophagy. This possibility seems more likely than glucose induction of these enzymes since glucose catabolites are known to induce the inactivation of FBP in *S. cerevisiae* (Holzer, 1976; Chiang and Schekman, 1991; Schork et al., 1994).

In order to rule out the possibility that the phenotype being tested was due to a generalized defect in vacuolar degradation, two analyses were undertaken. First, the activities of the important vacuolar proteases PrA and CPY (see Chapters 1 and 3) were assayed in WDY1 and WDY2 and found to contain approximately the same levels of these enzymes as the wild type. Second, ultrastructural examinations of these two strains were carried out. It was expected that, if the vacuole had been rendered proteolytically inactive while autophagic sequestration was still operative, then peroxisomes would accumulate in the vacuole as was shown to be the case in vacuolar proteinase deficient mutants (see Fig. 3-10). Indeed, based on the standard glucose adaptation assay, the vacuolar protease mutants are autophagy-deficient (see Fig. 3-10) due to their inability to degrade sequestered components. However, as mentioned previously, the purpose of this study was to understand how and where cellular components are sequestered, not the act of degradation per se

and therefore, if *gsa* mutant strains were found to be deficient in vacuolar proteolysis, these strains would not be further studied.

Cells of the strain WDY2 were fixed for electron microscopic examination using the standard potassium permanganate protocol at 0, 1.5, and 5 h of glucose adaptation (Fig 4-4). As a check that the cultures being sampled actually contained autophagy-defective strains, cell-free extracts were prepared at 0 and 6 hours of glucose adaptation from these same cultures and assayed for AOX activity. The biochemical results indicated that the cells which were fixed were mutant (data not shown). Just prior to initiation of glucose adaptation, the morphology of WDY2 (Fig. 4-4A) was indistinguishable from wild type cells. These cells contained peroxisomes in clusters as usual, typical mitochondria, and nuclei.

Instances of autophagic sequestration events were not detected in WDY2 at 1.5 of glucose adaptation (Fig. 4-4B) at a time point when peroxisomes are commonly seen being taken up by microautophagy in the wild type cells (see Fig. 3-6). After 5 h of glucose adaptation, microautophagy was still not observed in WDY2, but examples of macroautophagic sequestration were observed (Fig. 4-4C). In this micrograph, the vacuole appears to have fused with a peroxisome-containing autophagosome. The vacuolar membrane is continuous with the outer autophagic vacuolar membrane (see arrow in Fig. 4-4C) in which a peroxisome, still encased in extra membranes layers is observed. This process is similar to events described during glucose adaptation in *H. polymorpha* (see Fig. 3-7; Veenhuis et al., 1983). Macroautophagy has not been observed in wild

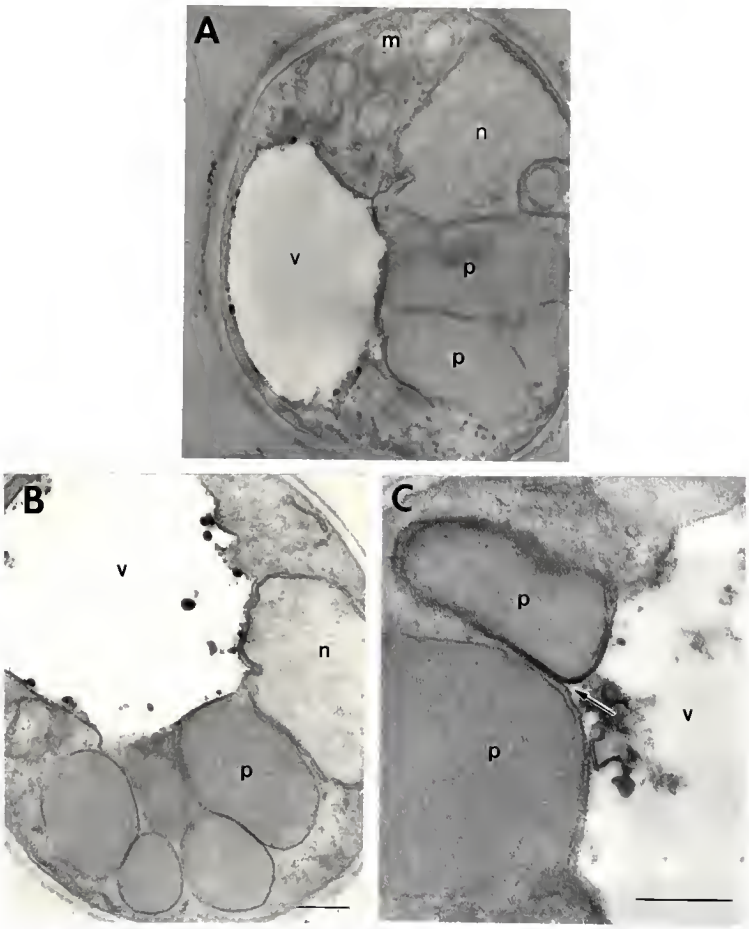

Figure 4-4. Ultrastructural examination of glucose adaptation in WDY2. WDY2 was grown to stationary phase in methanol and then glucose was added to a final concentration of 2.0% to initiate glucose adaptation. Samples were removed just prior to glucose addition (A), at 1.5 h after glucose addition (B), and 5 h after glucose addition (C). Arrow in C points to vacuole membrane which is continuous with autophagosome membrane surrounding peroxisome. p = peroxisome; m = mitochondrion; n = nucleus; v = vacuole; Bar = 0.5 μm.

type *P. pastoris* cells during glucose adaptation previously. It is possible that a null mutation of the GSA2 gene (that may be present in WDY2) completely blocks microautophagy. The minor decrease in peroxisomal proteins that occurs during 6 h of glucose adaptation (see Fig. 4-3A) may then be due to macroautophagy, which may be only slowly initiated during glucose adaptation and, thus, serves as a backup mechanism of peroxisome removal. In WDY2, after 5 h of glucose adaptation, peroxisome clusters were observed outside the vacuole in most of the cells examined (Fig. 4-4C). This is contrary to the case in the wild type cells, in which peroxisomes are only rarely seen outside the vacuole after only 3 h glucose adaptation (data not shown).

Taken together, the data suggest that the vacuoles WDY2 are proteolytically active, however, microautophagic sequestration into the vacuole is hindered. It appears that, in WDY2, the macroautophagy pathway is still operational but is not rapidly initiated during glucose adaptation. Identification of the mutated gene responsible for the defect in WDY2 is currently being pursued (see Appendix).

Chapter Summary

The present chapter describes the successful development of methods for production and isolation of mutant strains of *P. pastoris* which are deficient in the microautophagic sequestration of methanol-induced peroxisomes. A rapid and simple screen for candidate *gsa* (glucose-induced selective autophagy) mutants,

based on the retention of AOX activity after incubation on glucose (see Fig. 4-1), allowed the screening of hundreds of thousands of colonies within a few weeks from which 15 clones have been tentatively verified as *gsa* (see Fig. 4-2) out of a total of 48 positive colonies.

Two complementation groups including three alleles have been identified which have been shown to be significantly inhibited in the degradation of peroxisomes and cytosolic FDH (see Fig. 4-3) and fail to sequester peroxisomes by microautophagy (see Fig. 4-4). WDY1 and WDY2 have also been shown to contain functional vacuole proteases PrA and CPY, suggesting that the vacuole itself is proteolytically active but is unable to sequester peroxisomes and FDH. Ultrastructural biochemical analyses of WDY2 (see Fig. 4-3, 4-4) suggest that the normal GSA2 gene product normally functions downstream of the signaling step (see Fig. 6-4). Since glucose induced some degree of AOX degradation in 6 h, and macroautophagic sequestration of peroxisomes is initiated, then it appears that the signal for the presence has been received by the cells. The vacuole is not seen to cup around clusters of peroxisomes, suggesting that either the vacuole cannot recognize the peroxisomes as destined to be degraded, or else is unable to change shape to engulf the peroxisomes. The molecular basis of these two phenomena is unknown but may involve a guanine nucleotide-binding protein-mediated recognition event, and the vacuolar shape change could be dependent upon cytoskeletal elements.

In summary, *P. pastoris* has been shown to be a workable model for the genetic analysis of microautophagy. The power of yeast genetics, amply illustrated many times in *S. cerevisiae*, can be brought into play in *P. pastoris*, with relatively minor modifications (Gould et al., 1992). Work on the cloning and sequencing of the defective gene in WDY2 is ongoing (see Appendix). Finally, the present work has demonstrated procedures which should lead to the analysis of the molecular mechanisms of selective autophagy.

CHAPTER 5
MULTIPLE PATHWAYS OF AUTOPHAGY IN *PICHIA PASTORIS*

Introduction

The addition of glucose to methanol-induced cultures elicits the selective degradation of peroxisomes in *P. pastoris* by microautophagy (see Fig. 3-1, 3-2, 3-3, 3-4, 3-6, 3-10). It was of interest then, to determine whether different environmental conditions could be manipulated so as to invoke other forms of autophagy that could be studied in *P. pastoris*. Investigations relating to the effects of adapting methanol-induced yeasts to ethanol suggest that this carbon source also mediates the degradation of peroxisomes (Bormann and Sahm, 1978; Hill et al., 1985). The mode of degradation of peroxisomes induced by ethanol has not been investigated on the ultrastructural level in any of the methylotrophic yeasts. Therefore it is not known if the peroxisomes are sequestered by macroautophagy, microautophagy or a unique process.

Culture of cells in media without a source of nitrogen has been shown to increase the nonspecific vacuolar degradation of cellular components in the yeast *S. cerevisiae* (Takeshige et al., 1992; Teichert et al., 1989). Nitrogen starvation of vacuolar protease mutant strains of *S. cerevisiae* leads to the accumulation of undegraded cellular components in the vacuole

(Baba et al., 1994; Takeshige et al., 1992). Evidence was presented suggesting that these cellular components are nonselectively sequestered into autophagosomes that enter the vacuolar lumen and are degraded there. This form of autophagy is similar to macroautophagy as carried on by mammalian cells.

Inasmuch as different forms of selective autophagy are initiated by glucose in two different species of methylotrophic yeasts (see Chapter 3) and evidence exists for nonselective autophagy induced by nitrogen starvation in *S. cerevisiae*, I investigated ethanol adaptation and starvation in *P. pastoris*. This was accomplished using the tools which have been validated in the foregoing parts of the study, including biochemical evaluations of enzyme activity and protein; ultrastructural analysis; and the use of the protease and *gsa* mutants.

Ethanol Adaptation

Characterization of Ethanol Adaptation in Wild Type Cells

Experiments were performed to determine the response of methanol-induced *P. pastoris* to the addition of ethanol to a 0.5% final concentration, in the same type of protocol as utilized to measure glucose adaptation. In contrast to the case of glucose adaptation, ethanol causes a more rapid decrease in AOX activity. In the first hour of adaptation, ethanol induces a 50% decrease in AOX activity while this activity remains unchanged for the first hour of glucose adaptation (Fig. 5-1A, compare to Fig. 3-4). Another difference between glucose

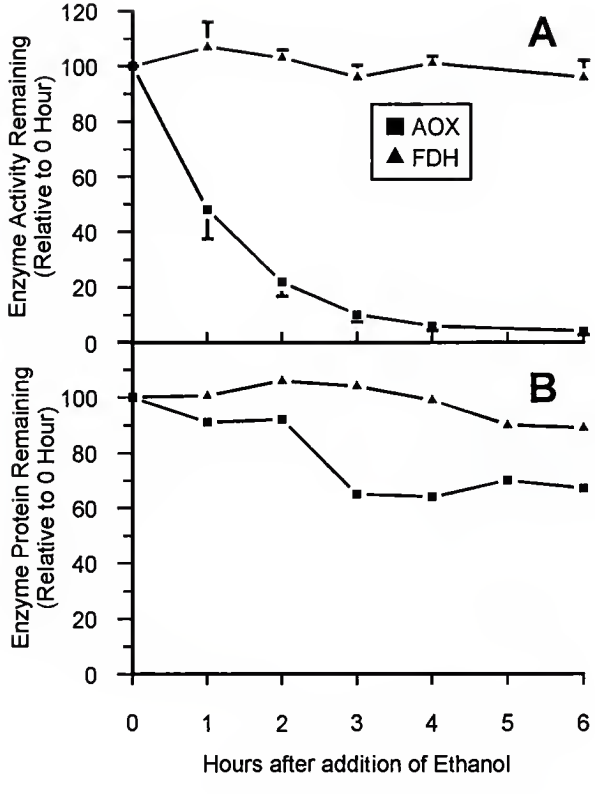

Figure 5-1. Degradation of peroxisomal and cytosolic enzymes during ethanol adaptation. *Pichia pastoris* cells (GS115) were cultured in methanol induction medium until stationary at which time ethanol was added to begin adaptation. Cell-free extracts were prepared at selected times and peroxisomal alcohol oxidase (AOX) and cytosolic formate dehydrogenase (FDH) activities assayed (A). The values represent the average ± s.e.m. of six determinations. Protein levels of AOX and FDH in extracts of ethanol adapting cells were analyzed by laser densitometry of immunoblots (B).

and ethanol adaptation is illustrated by comparing the fate of AOX protein. While glucose induces the roughly concurrent decrease of AOX activity and protein, ethanol causes a much more rapid decrease in AOX activity than of AOX protein (compare Fig. 5-1B, closed squares, to the immunoblot data in Figure 3-4, inset). As is the case for glucose adaptation (see Fig. 3-1), another marker for methanol-induced peroxisomes, dihydroxyacetone synthase (DHAS), is degraded at the same rate as AOX during ethanol adaptation based on laser densitometric scans of Coomassie stained polyacrylamide gels (data not shown). AOX protein is undetectable by immunoblot and on Coomassie stained gels in methanol-induced cells which have undergone 24 h of ethanol or glucose adaptation. Therefore, while the degradation rate of peroxisomal proteins is different under these two conditions, they are eventually degraded in both cases.

The more rapid reduction of AOX activity during ethanol adaptation compared to that observed during glucose adaptation suggested that some other event must take place before the initiation of peroxisome degradation during glucose adaptation. One possibility is that synthesis of new protein must take place before glucose-induced peroxisome degradation can commence. Therefore, I tested various concentrations of cycloheximide for inhibition of protein synthesis in *P. pastoris*. Protein synthesis as measured by the incorporation of [^{35}S]-methionine/cysteine into cellular proteins was inhibited by 40% at cycloheximide concentration of 0.1 mg/mL and was ~90% inhibited at 1.0 mg/mL. Next, the effect of 1.0 mg/mL cycloheximide on ethanol and glucose adaptation in *P. pastoris* was examined (Fig. 5-2A). Inhibition of protein

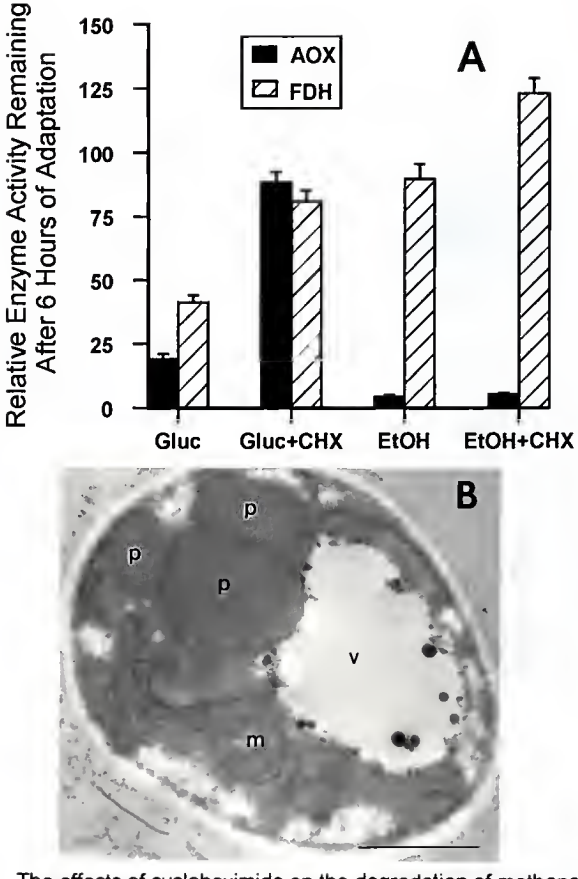

Figure 5-2. The effects of cycloheximide on the degradation of methanol-induced enzymes and peroxisomes during ethanol and glucose adaptation. Methanol-induced GS115 cells were adapted to ethanol or glucose in the absence or presence of cycloheximide (1 mg/mL). Alcohol oxidase (AOX) and formate dehydrogenase (FDH) activities were determined in cell-free extracts obtained at 0 hours and 6 hours of adaptation. The values represent activities (mean + s.e.m.) measured at 6 hours and reported relative to that measured at 0 h (A). GS115 cells that had been adapted to glucose for 6h in the presence of cycloheximide were fixed and embedded for ultrastructural examination as described in Materials and Methods (B). p = peroxisome; v = vacuole; m = mitochondrion; Bar = 0.5 μm.

synthesis effectively blocked glucose-induced peroxisome and FDH degradation but did not affect the ethanol-induced decrease in AOX activity. In the methylotrophic yeast *Candida boidinii*, ethanol mediated peroxisome degradation is also not dependent on protein synthesis (Bormann and Sahm, 1978). Ultrastructural examination of cells after 6 h of glucose adaptation in the presence of cycloheximide revealed clusters of peroxisomes outside the vacuole (Fig. 5-2B). The vacuole was never seen encircling peroxisomes as occurs in the absence of cycloheximide (see Fig. 3-6).

Another biochemical indication of differences mediated by glucose and ethanol in methanol-induced *P. pastoris* was noted. While glucose induces a large decrease in the cytosolic methanol assimilation enzyme FDH during the first 6 h of adaptation (see Fig. 3-4), FDH activity and protein levels remain constant during 6 h of ethanol adaptation (Fig. 5-1, closed triangles). Similarly to glucose, addition of ethanol to methanol-induced cells does not cause a decrease in mitochondrial CCO activity or F1β protein (data not shown). These data suggest a difference in selectivity between the two pathways, i.e., glucose selects peroxisomal and cytosolic methanol utilization proteins for degradation while ethanol selects only peroxisomes.

Taken together, these data suggest that the decrease in AOX activity observed during ethanol adaptation indeed occurs by a different pathway than the degradative inactivation of peroxisomes mediated by glucose. One possibility that presents itself is that the AOX is inactivated and/or degraded in a

compartment upstream of the vacuole during ethanol adaptation. If, for instance, this event takes place while the peroxisomes are still in the cytoplasm , the rapidity with which inactivation follows ethanol addition could be accounted for.

Investigation of the Site of Inactivation of AOX During Ethanol Adaptation

The investigation of where AOX is inactivated was implemented by utilizing two separate strategies, ultrastructural examination of *P. pastoris* during ethanol adaptation, and biochemical assays of AOX activity in protease mutant cells in which the vacuoles have been shown to be proteolytically inactive. Wild type cells after 1 h of ethanol adaptation were fixed with potassium permanganate as described above and examined by transmission electron microscopy. After 1 h of ethanol adaptation, peroxisomes were seen sequestered into autophagosomes by membrane layers of unknown origin (Fig. 5-3A). At this time point, peroxisomes were observed either sequestered or still unsequestered. After this first hour of ethanol adaptation, AOX activity levels declined by 50 % (see Fig. 5-1A,closed squares) but AOX protein levels were not changed (see Fig. 5-1B, closed squares). The autophagosomes are frequently observed apparently fusing with the degradative vacuole (Fig. 5-3B) and are often seen inside the vacuole, usually still retaining extra membrane layers, presumably residual components of the limiting membranes of the autophagosome (Fig. 5-3C). This is in contrast to the microautophagic manner of peroxisome sequestration observed in *P. pastoris* during glucose adaptation (see Fig. 3-6) but very similar to macroautophagy of peroxisomes in *H. polymorpha* (see Fig. 3-7; Veenhuis et al., 1983). In any case, these data

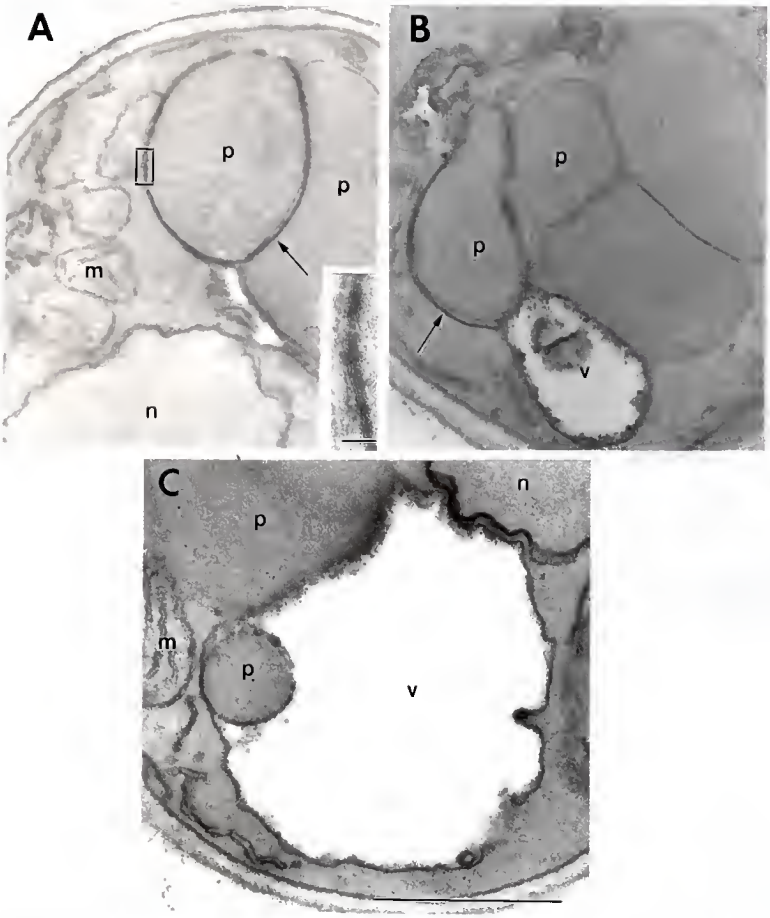

Figure 5-3. Morphological characterization of ethanol-induced peroxisomal degradation in *P. pastoris*. *P. pastoris* was grown in methanol medium until stationary growth was achieved. Ethanol was then added to the cultures to a final concentration of 0.5% then incubated for one additional hour and potassium permanganate fixed as described in Chapter 2. Inset in A corresponds to boxed area; arrows in A and B delineate limiting membranes of autophagic vacuole containing a sequestered peroxisome. p = Peroxisome; v = Vacuole; m = Mitochondrion; n = nucleus; bar = 0.5 µm; Inset Bar = 25 nm.

suggest ethanol does induce the rapid vacuolar uptake of peroxisomes. However rapid inactivation of AOX before the peroxisomes have entered the vacuole remains a possibility.

Ethanol adaptation of methanol-induced cells was also carried out in protease mutant cells, lacking functional PrA and PrB. After six hours of ethanol adaptation AOX activity was virtually unchanged in these cells which are not able to degrade cellular components in the vacuole (Fig. 5-4A). Indeed, electron microscopic examination of protease mutant cells after 6 h of ethanol treatment revealed an accumulation of peroxisomes in the vacuole (Fig. 5-4B), similar to that seen during glucose adaptation (see Fig. 5-6B). These two lines of evidence suggest that the vacuole is the site of AOX inactivation and degradation during ethanol adaptation. Another possibility that can not be ruled out is that AOX is inactivated while it is in the autophagosome before it has fused with the vacuole. This is only possible if the autophagosomal membranes contain some PrA/PrB-dependent AOX inactivation activity, in which case, the autophagosomal membranes may be vacuolar in origin since these activating endoproteases are not known to be active outside the vacuole.

Questions have been left unresolved by these analyses: what prevents the entire peroxisome from being rapidly degraded in the vacuole in normal cells in which, once things are sequestered there, degradation usually appears to be rapid and nonspecific. Other questions which present themselves are: How is the AOX inactivated? Why is it protease dependent? One hypothesis is that, since the peroxisomes sequestered into the vacuole usually are observed to be

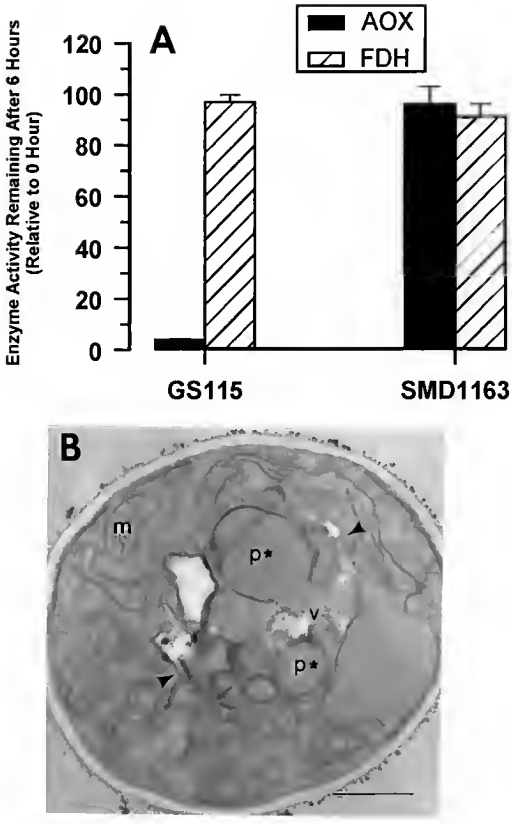

Figure 5-4. Proteinase mutants are not able to degrade methanol-induced components during ethanol adaptation. Parental (GS115) and protease mutant (SMD1163) cells were grown in methanol then adapted to ethanol. Alcohol oxidase (AOX) and formate dehydrogenase (FDH) activity were determined at 0 hours and 6 hours of ethanol adaptation (A). The values represent the average + s.e.m. of at least three determinations done at 6 hours of adaptation and is reported as a percentage of that measured at zero hour. At 6 hours of ethanol adaptation, the mutant cells were fixed and processed for ultrastructural examination (B). Intact peroxisomes (*) were observed within the vacuoles (v) of proteinase mutant cells (B). Arrowheads indicate the perimeter of the yeast vacuole. p = peroxisome; m = mitochondrion; Bar = 0.5 μm.

still encased in residual autophagosome membranes (see Fig. 5-3C), the contents may not be susceptible to rapid, generalized degradation. AOX activity has been shown to depend on the presence of FAD bound as a prosthetic group (Bruinenberg et al., 1982). In the case of ethanol adaptation of peroxisome assembly mutants of *H. polymorpha*, it was shown that AOX activity decreased much faster than AOX protein (van der Klei et al., 1991; see Chapter 1). The AOX activity was shown to decrease at the same rate as the decrease in AOX-bound FAD. Therefore, the possibility exists that PrA, PrB, and/or hydrolases normally activated by these endoproteases are able to enter the autophagosome-encased peroxisomes in amounts sufficient to cause the AOX homo-octameric complexes to release the covalently bound FAD but are not able to rapidly degrade the entire contents.

Ethanol Adaptation in *gsa* Mutants

A final indication of the divergence of the autophagy pathways induced by ethanol and glucose, was the behavior of the *gsa* mutants which were selected on the basis of inability to degrade peroxisomes in response to glucose. Both *gsa* complementation groups inactivated AOX at the wild type rate in response to ethanol while being severely deficient in glucose-induced peroxisome degradation (see Fig. 4-3A). A comparison of the ethanol adaptation data between parental GS115 and autophagy-deficient WDY2 is shown (Fig. 5-5). The mutant strain degrades AOX in response to ethanol at the wild type rate, suggesting that the ability to degrade peroxisomes in response to ethanol is unimpaired in this glucose-induce selective autophagy mutant.

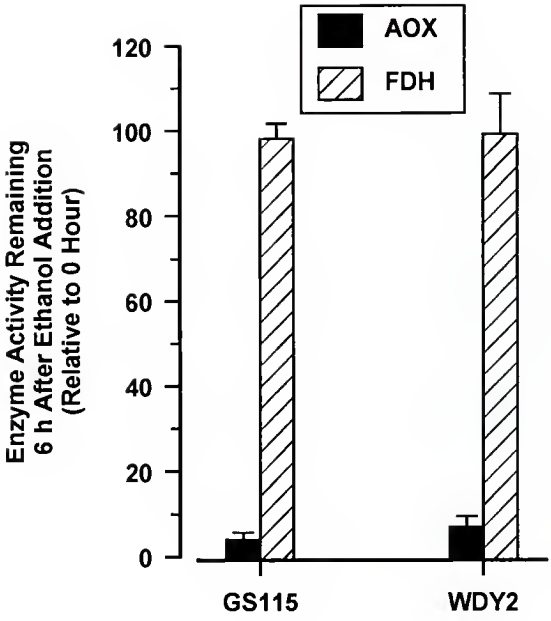

Figure 5-5. Comparison of ethanol adaptation in GS115 and WDY2. Enzyme assays were conducted on cell-free extracts prepared from parental *P. pastoris* (GS115) mutant strain WDY2 at 0 and 6 h of ethanol adaptation. The results are expressed as the mean + s.e.m. of the percentage of alcohol oxidase (AOX) and formate dehydrogenase (FDH) activity present at 6 h relative to that present at 0h.

Nitrogen Starvation

Ultrastructural and biochemical analyses of nitrogen starvation in *S. cerevisiae* revealed that this treatment induces the autophagic delivery of peroxisomes, mitochondria, ribosomes, and nondescript vesicular structures to the vacuole for degradation (Baba et al., 1994; Takeshige et al., 1992; Teichert et al., 1989). Indeed, starvation for histidine was shown to cause an increase in protein degradation in *P. pastoris* (see Fig. 3-8). Therefore, I examined the effects of nitrogen starvation on the wild type strain of *P. pastoris* (GSA115) and a strain which lacks the putative vacuolar proteases PrA and PrB (SMD1163) to determine whether autophagy can be induced in *P. pastoris* by this treatment. *P. pastoris* cells were grown to stationary phase in nitrogen- and glucose-containing medium then switched to a medium lacking a nitrogen source but still containing glucose and other essential nutrients. After 3 h of nitrogen starvation the cells were harvested and fixed with potassium permanganate as described (Chapter 2). The vacuoles of normal cells that were cultured in nitrogen-rich medium (data not shown) or nitrogen-depleted medium (Fig. 5-6A) were largely empty with infrequent vesicular bodies. Likewise, few vesicular bodies were present in proteinase mutant cells grown in the presence of nitrogen (Fig. 5-6B). However, numerous profiles of nondescript vesicular bodies accumulated in the vacuoles of nitrogen-starved SMD1163 (Fig. 5-6C). Intact mitochondria were also observed within some vacuoles (Fig. 5-6C, inset). These observations indicate that nitrogen starvation

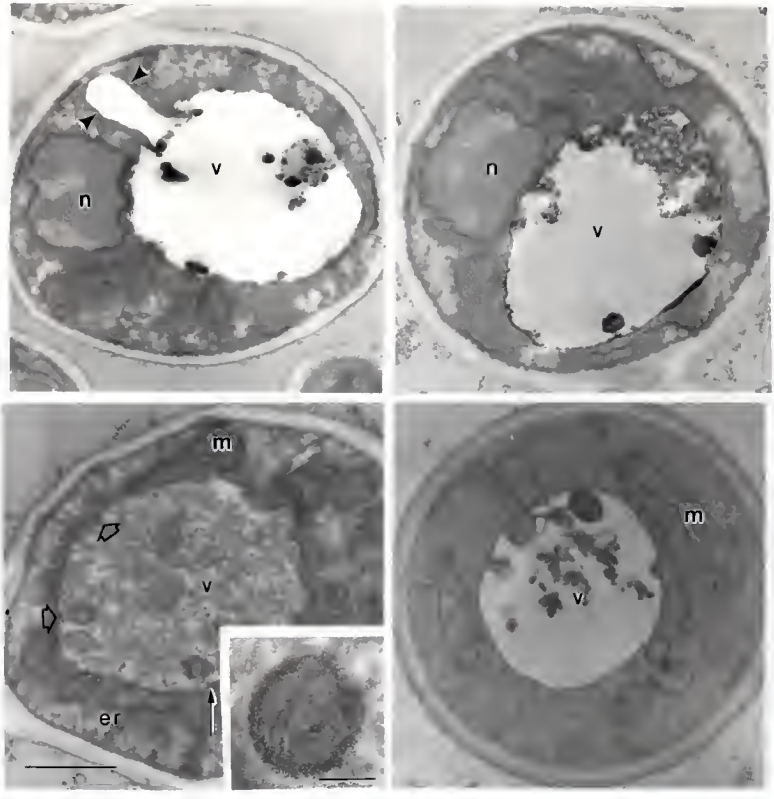

Figure 5-6. Effects of nitrogen starvation on parental and proteinase mutant *P. pastoris*. GS115 (A) and SMD1163 (B,C,D) cells were grown to stationary phase in YPD medium and then resuspended in media either lacking (A,C,D) or containing (B) a nitrogen source. The cells were incubated for 3 hours in these media then processed for electron microscopy. The arrowheads in (A) indicate a large finger-like protrusion. Open arrows indicate microautophagic pits (C) and an ongoing microautophagic event is indicated by the solid arrow in (C). Nitrogen starved protease mutant cells were cultured in the presence of 1.0 mg/mL cycloheximide in (D). v = vacuole; m = mitochondrion; n = nucleus; er = endoplasmic reticulum; Bar: 0.5 μm; Inset Bar = 0.1 μm.

induces the sequestration of cytoplasmic organelles into the vacuole. These data also provide further evidence that functional PrA and PrB are required for the normal degradative activity of the vacuole.

Comparison of Vacuolar Uptake of Cellular Components

Morphological evidence, utilizing vacuolar protease mutant cells, suggests that the vacuole is indeed the site of degradation of cellular components during nitrogen starvation (see Fig. 5-6C) as it is during glucose (see Fig. 3-10) and ethanol (see Fig. 5-3) adaptation. Nitrogen starvation causes the vacuole to extend finger-like projects (see Fig. 5-6A) and form pits (see Fig. 5-6C) which may assimilate portions of cytoplasm into the vacuole and cause their degradation. Other indications of the active nature of the vacuolar membrane during nitrogen starvation are seen (arrow in Fig. 5-6C) which suggest the microautophagic uptake of cytoplasmic components into the vacuole. Thus, while it appears that glucose adaptation of methanol-induced cells and nitrogen starvation both induce microautophagy, ultrastructure of the microautophagic events are somewhat different (compare Fig. 3-6 to Fig. 5-6). Glucose appears to cause the entire vacuole to change shape as a whole and surround and take up large peroxisome clusters (see Fig. 3-6), but nitrogen starvation appears to induce restricted portions of the vacuolar membrane to independently sequester relatively small portions of cytoplasm (see Fig. 5-6).

Ethanol appears to induce a very different form of vacuolar uptake which involves the macroautophagic sequestration of individual peroxisomes into

autophagosomes which subsequently fuse with and deposit their contents into the vacuole (see Fig. 5-3). Consequently, it appears that three separate pathways of autophagy may be demonstrated in *P. pastoris*: macroautophagy induced by ethanol and two distinct forms of microautophagy induced by nitrogen starvation or glucose.

To examine the consequences of stoppage of protein synthesis in nitrogen starved cells, vacuolar protease mutant *P. pastoris* cells (SMD1163) were grown to stationary phase in complete medium containing glucose (YPD), washed and resuspended in nitrogen-free medium containing 1.0 mg/mL cycloheximide. After 3 additional hours of incubation cells were harvested, fixed with potassium permanganate and viewed by transmission electron microscopy (Fig. 5-6D). Inhibition of protein synthesis did not cause the abundant vacuolar accumulation of cytosolic components observed in vacuolar protease mutant cells deprived of nitrogen in the absence of cycloheximide (compare Fig. 5-6C and D). Cycloheximide-treated cells also did not exhibit the invaginations and protrusions of the vacuolar membrane (Fig.5.6D), suggesting a decrease in microautophagic capacity similar to that seen in cycloheximide-treated cells undergoing glucose adaptation (see Fig. 5-2B).

The observable effect of blocking protein synthesis in cells undergoing glucose adaptation and nitrogen starvation is that the vacuolar membrane does not change shape to sequester cytoplasmic components. Therefore, it is possible that the changes in shape of the vacuolar membrane necessary for

microautophagy depend on the continuous synthesis of some protein(s).

The effect of the protein synthesis inhibitor cycloheximide on the starvation mediated loss of peroxisomal, cytosolic, and mitochondrial marker enzymes was also evaluated (Fig. 5-7). To accomplish this, peroxisomal, cytosolic, and mitochondrial enzyme activities were measured before and after nitrogen starvation wild type *P. pastoris* cells in the presence and absence of cycloheximide (Fig. 5-7). Inhibition of protein synthesis efficiently blocked the inactivation of these enzymes brought about by starvation conditions in the absence of cycloheximide. Previously, cycloheximide was shown to prevent glucose-induced microautophagy and degradation but had no effect on ethanol-induced macroautophagy (see Fig. 5-2). It may be concluded that both forms of microautophagy require protein synthesis while ethanol-induced macroautophagy does not.

Comparison of Selectivity During Nitrogen Starvation and Glucose and Ethanol Adaptation

Degradation induced by nitrogen starvation of *P. pastoris* cells incubated in methanol or glucose media appears to lack specificity since peroxisomal, cytosolic, and mitochondrial proteins are all degraded (Fig. 5-7). This is in contrast to the case during glucose adaptation of methanol-induced *P. pastoris* cells, during which the degradation process appears to be limited to methanol-induced cytosolic proteins (FDH) and peroxisomes (see Fig. 3-4, 3-6, 3-10). Indeed, a different selectivity is exhibited by *P. pastoris* cells during ethanol

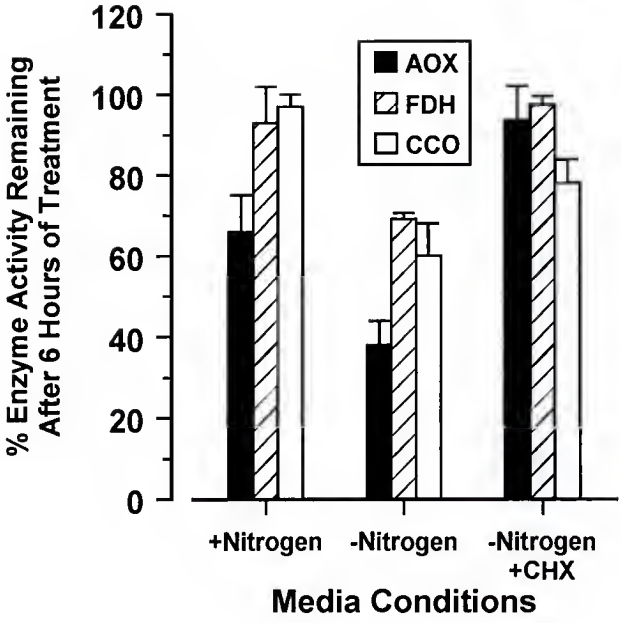

Figure 5-7. Effect of nitrogen deprivation on peroxisomal, cytosolic, and mitochondrial enzymes. Wild type *P. pastoris* (GS115) was grown to stationary phase in methanolic media, then switched either to medium lacking a carbon source (+ Nitrogen) or lacking a carbon and nitrogen source in the absence (- Nitrogen) or presence of 1.0 mg/mL cycloheximide (-Nitrogen+CHX). Cell-free extracts were produced from samples taken at the beginning and end of 6 h of treatment and were assayed for alcohol oxidase (AOX), formate dehydrogenase (FDH), and cytochrome c oxidase (CCO) activity. The values indicated are the means + s.e.m. of the amounts of enzyme activity present at 6 h of treatment relative to that present at 0 h.

adaptation in which peroxisomes are rapidly sequestered into vacuoles (see Fig. 5-3) but cytosolic FDH is not degraded during the first 6 hours of adaptation (see Fig. 5-1). Mitochondrial F1β is also not degraded during ethanol adaptation, suggesting that ethanol elicits the specific degradation of peroxisomes but not cytosolic proteins or mitochondria.

Morphological evidence also suggests that the autophagic pathways elicited by glucose and ethanol are selective since only peroxisomes are observed to be sequestered (Fig. 3-6, 5-3). Evidence for nonselective autophagy in *P. pastoris* is provided by ultrastructural analyses of nitrogen-starved vacuolar protease mutants (see Fig 5-6). Close examination of the cytosolic components which have accumulated in the vacuole reveals the presence of many nondescript vesicular structures, many of which contain multiple internal membranes suggesting that they derive from mitochondria. Definite mitochondrial profiles are also occasionally seen (inset, Fig., 5-6C). Taken together, these data suggest that starvation causes the nonselective degradation of cellular components.

Evaluation of Autophagy Induced by Nitrogen Deprivation in *gsa* Mutants

Autophagy of peroxisomes induced by nitrogen starvation, ethanol, and glucose was studied in *gsa* mutants to get a clearer picture of the interrelationships between the three postulated pathways. AOX activity was measured in methanol-induced cultures of GS115, WDY1, and WDY2 before and after 6 h nitrogen deprivation, glucose, or ethanol adaptation (Fig. 5-8). A

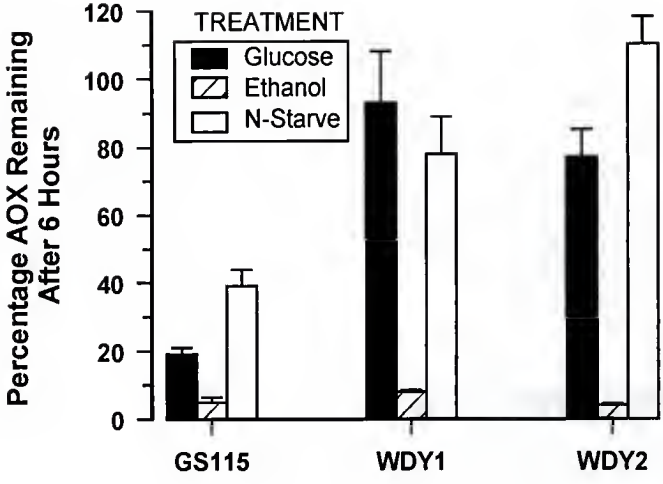

Figure 5-8. Comparison of autophagy of peroxisomes induced by glucose, ethanol, and nitrogen deprivation in wild type and *gsa* mutant *P. pastoris* strains. Cells of the *P. pastoris* strains GS115, WDY1, and WDY2 were grown to stationary phase then either glucose (solid bars), or ethanol (hatched bars) were added or the cells were switched to nitrogen-free medium (N-Starve; open bars) and incubated for 6 h. Samples were removed from cultures before and at the end of 6 h treatment, then cell-free extracts prepared which were assayed for alcohol oxidase (AOX). Results are mean + s.e.m. of AOX activity after 6 hours treatment relative to that at the start of treatment.

large decrease of AOX activity was observed in response to six hours of nitrogen deprivation and glucose adaptation in wild type cells but this effect was strongly inhibited in both *gsa* mutant strains. In contrast, the effect of ethanol treatment on AOX was not affected by the *gsa* mutations. These data strongly suggest that the products of the genes which are mutated in WDY1 and WDY2 are required for both forms of microautophagy but not for ethanol-induced macroautophagy.

Chapter Summary

Ultrastructural examination of the wild type and protease mutant strains of *P. pastoris* has provided strong evidence that ethanol induces the macroautophagic delivery of methanol-induced peroxisome into the vacuole where AOX is rapidly inactivated but slowly degraded (see Fig. 5-1, 5-3, 5-4). The data presented in this chapter demonstrates the existence of a pathway for the uptake of peroxisomes into the vacuole of *P. pastoris* distinct from microautophagy as induced by glucose. The lines of evidence for this conclusion are 1) more rapid onset of vacuole-mediated disappearance of AOX activity during ethanol adaptation (compare Fig 3-4 to Fig. 5-1A); 2) the protein synthesis inhibition-sensitive nature of glucose-induced autophagy but not ethanol-induced autophagy (see Fig. 5-2); 3) the ultrastructural data showing the microautophagic sequestration of peroxisomes in response to glucose in contrast to the macroautophagic sequestration of peroxisomes induced by

ethanol (compared Fig. 3-6 to Fig. 5-3); finally, the *gsa* mutants, defective in microautophagy (see Fig. 4-3a, 4-4) are fully functional in the vacuole-mediated inactivation of AOX in response to ethanol (see Fig. 5-5).

Data were presented that suggest two forms of microautophagy and one form of macroautophagy can be initiated in *P. pastoris*. The evidence presented indicates both differences and similarities between glucose-induced and nitrogen starvation-induced microautophagy. The similarities are: 1) cytoplasmic components are sequestered by invaginations of the vacuolar membrane; 2) both processes are dependent on protein synthesis; 3) two different *gsa* mutations affect both pathways; and 4) the site of degradation is the vacuole. The differences are: 1) glucose induces more generalized changes in the vacuolar membrane, causing the overall shape of the vacuole to change to engulf large portions of cytosol, but nitrogen starvation causes more limited invaginations which sequester smaller cytosolic sectors; 2) glucose induces the selective degradation of methanol-induced components while nitrogen starvation causes nonspecific uptake and degradation of cytoplasm, including mitochondria, peroxisomes, and soluble proteins; and 3) the nutrient signals which activate the pathways. The macroautophagic pathway induced by ethanol is distinct from each of these pathways both in morphological events and selectivity of objects to be degraded. A comparison of the pathways of autophagy induced by the various stimuli is presented in Table 5-1.

In conclusion, the protocols and yeast strains developed or characterized in the present study have proven invaluable for detection of three distinct autophagic pathways in *P. pastoris* (see Table 5-1). Further investigations utilizing the power of yeast genetics and cell biology demonstrated herein by manipulation of *P. pastoris* should rapidly yield further unique insights into the mechanisms of both microautophagy and macroautophagy.

Table 5-1. Comparison of the characteristics of autophagy induced by various stimuli

STIMULUS	MICRO/ MACRO ?[a]	CHX-SENSITIVE ?[b]	gsa-SENSITIVE ?[c]	SELECTIVE ?[d]	SITE OF DEG ?[e]
GLUCOSE ADAPTING	MICRO	YES	YES	YES	VAC
ETHANOL ADAPTING	MACRO	NO	NO	YES	VAC
NITROGEN STARVED	MICRO	YES	YES	NO	VAC

[a] Does the stimulus cause primarily microautophagy or macroautophagy?
[b] Is autophagy induced by the stimulus blocked by 1.0 mg/mL cycloheximide?
[c] Is autophagy induced by the stimulus inhibited in mutant strains WDY1 and WDY2?
[d] Does autophagy induced by the stimulus selectively sequester certain cytoplasmic components?
[e] What is the cellular location for the final degradation event (VAC = vacuole)?

CHAPTER 6
CONCLUSIONS, MODELS, AND PROSPECTS

Catabolite Inactivation

It has long been recognized that microbial cells growing on nonglucose carbon sources respond to the addition of glucose to their growth media by repressing the synthesis of certain enzymes at the level of transcription and inactivating others by total degradation or modification (Holzer, 1976; Holzer and Purwin, 1986). It was ascertained that catabolites of glucose and not glucose itself were responsible for the repression and inactivation of glucose-sensitive enzymes so the terms "catabolite repression" and "catabolite inactivation" were proposed for these effects, respectively. Investigations revealed that enzymes repressed/inactivated by glucose catabolites in yeast "are capable of converting their substrates to metabolites which the cell can also obtain independently and more readily by the metabolism of glucose" (Holzer, 1976). Several cytosolic enzymes have been determined to undergo catabolite inactivation (see Chapter 1).

In the methylotrophic yeasts *H. polymorpha* and *Candida boidinii*, catabolite repression/inactivation of methanol-induced peroxisomes and cytosolic enzymes in response to glucose or ethanol has been observed (Bormann and Sahm, 1978; Bruinenberg et al., 1982; Hill et al., 1985; Veenhuis

et al., 1978, 1983). These reports constitute the first examples of catabolite inactivation of entire organelles. However in none of these cases has the degradation of methanol-induced elements been measured directly. Rather, decreases in total cellular levels of AOX, DHAS, catalase, or FDH have been measured without formally evaluating the possible counterbalancing phenomena of protein synthesis and protein degradation. In this study, I directly measured the synthesis and degradation of the peroxisomal proteins AOX and DHAS (see Fig. 3-2). These data show that the addition of glucose to methanol-induced cultures causes the rapid degradation of the constituent proteins of peroxisomes. These data indicate that AOX synthesis is very low in stationary phase in methanol compared to exponential phase and is not further decreased after the addition of glucose. Therefore, any decreases in cellular levels of AOX protein is indeed due to degradation.

Two mechanisms for catabolite inactivation have been observed: total proteolysis which is assumed to occur either by autophagy in the vacuole or in the cytosolic proteasome (Chiang and Schekman, 1991; Schork et al., 1994); and post-translational modifications, such as phosphorylation or removal of prosthetic groups (Holzer and Purwin, 1986; Bruinenberg et al., 1982). It appears that both of these processes function in during glucose adaptation in methylotrophic yeasts. In *H. polymorpha* and *P. pastoris* during glucose adaptation, rapid degradative catabolite inactivation takes place (see Fig. 3-2, 3-4). However, during ethanol adaptation, AOX activity is rapidly lost in a PrA/PrB-

dependent manner (50% in the first hour, perhaps by removal of covalently bound FAD from homo-octameric AOX), while degradation occurs much more slowly (see Fig. 5-1).

The site of degradative catabolite inactivation for the enzymes known to undergo this process in *S. cerevisiae* is still a matter of controversy due to disagreement over the site of degradation of FBP. Schork et al. (1994) contend that FBP must be degraded by the proteasome since proteasome mutants are defective in degrading it. On the other hand, Chiang and Schekman (1991) say it must be degraded in the vacuole since PrA/PrB mutants fail to degrade FBP at the wild type rate in response to glucose and suggest the former researchers results are due to the pleiotropic phenotype of the proteasome mutants used (reply following Schork et al., 1994; Heinemeyer et al., 1991). The consensus seems to be that vacuolar protease mutants are reliable indicators of vacuole-mediated degradation in *S. cerevisiae* (Burlini et al., 1989; Funagama et al., 1985; Chiang and Schekman, 1991; Ammerer et al., 1986; Teichert et al., 1989).

P. pastoris strains have become available in which the genes homologous to PrA and/or PrB have been knocked out by homologous recombination. I have presented biochemical and morphological data which strongly suggests that PrA and PrB are necessary for normal levels of vacuolar proteolytic activity in *P. pastoris*. Strains lacking these proteases are unable to respond to deprivation of a required amino acid by increasing overall protein degradation (see Fig. 3-8); and undegraded cytoplasmic materials accumulate in PrA/PrB-deficient vacuoles

in response to nitrogen deprivation (see Fig. 5-6C) and glucose adaptation (see Fig. 3-9). In addition, strains lacking PrA exhibit lower that normal levels of CPY, a vacuolar exopeptidase which is activated by PrA-mediated proteolytic cleavage in *S. cerevisiae* (Ammerer et al., 1986; Teichert et al., 1987; Hirsch et al., 1992). These results provide strong evidence that PrA and PrB function in *P. pastoris* in a manner similar to the homologous enzymes in *S. cerevisiae*. Therefore, PrA/PrB-deficient strains were used to determine that the vacuole is the site of catabolite inactivation of methanol-induced peroxisomes during glucose and ethanol adaptation (see Fig. 3-9, 5-4).

The vacuole has been suggested to be the site of degradation of the cytosolic protein FDH during glucose adaptation (see Fig. 3-9). The mechanism whereby soluble proteins enter the vacuole is unknown; one study indicates that entry of FBP into the vacuole can be prevented by *sec* mutants which are blocked early in the secretory pathway. These data suggest that a protein which traverses the secretory pathway prior to being diverted to the vacuole must be synthesized before the vacuole is competent to take up FBP (Chiang and Schekman, 1991). In mammalian systems, the carrier-mediated transport of specific cytosolic proteins into lysosomes has been detected. This process involves the binding of proteins containing a pentapeptide sequence related to KFERQ (one-letter amino acid abbreviations) by a soluble receptor (prp73), a member of the hsp70 heat-shock protein family, which in some unknown manner facilitates the entry of the proteins into lysosomes (Chiang et al., 1989; Dice et

al., 1990; Terlecky et al., 1992). The existence of this system for vacuolar uptake of cytosolic proteins has not been confirmed in yeast.

In summary, I report here the first direct measurements of methanol-induced peroxisomal protein degradation and synthesis during glucose-mediated catabolite inactivation in *H. polymorpha* and *P. pastoris*. The yeast *P. pastoris* presents a model for both degradative catabolite inactivation (during glucose adaptation) and modificatory catabolite inactivation (during ethanol adaptation) of AOX. Evidence was presented that *P. pastoris* mutants lacking functional genes for the homologs of the *S. cerevisiae* vacuolar endoproteases are severely inhibited in vacuolar degradation. Data generated by the utilization of these mutants during studies of glucose and ethanol adaptation suggests that the vacuole is the site of inactivation of the cytosolic methanol assimilation enzyme FDH during glucose adaptation and peroxisomal proteins during both instances of inactivation induced by changes in carbon source.

Autophagy

As discussed in Chapter 1, autophagy in mammalian systems has been a well-recognized event for many years and has been extensively described relative to the morphological appearance of microautophagic and macroautophagic phenomena. Hormonal and nutritional regulation of degradation due to autophagy and of the appearance of autophagic events has also been investigated thoroughly (see Dunn, 1993, 1994 for reviews).

Unfortunately, the molecular mechanisms underlying the machinery of autophagy in mammals remain completely unexplored. One of the goals of the present project is to develop yeast models for autophagy in which the power of yeast genetics can be brought to bear on this problem.

Microautophagy

In mammals, microautophagy has been described mainly on the basis of lysosomal morphology. Two major types of microautophagic figures have been described in rats and mice (Mortimore et al., 1988): 1) type A lysosomes are secondary lysosomes containing internal vesicular structures which may arise from fusion of small cytoplasmic vesicles with primary lysosomes. Type A lysosomes have been distinguished from macroautophagic autolysosomes on the basis of unresponsiveness to amino acid suppression and lack of association with precursor autophagic vacuoles. It is not known how these bodies form. 2) Type R lysosomes exhibit flap-like protrusions or arrange themselves into cup shapes, presumably to capture nearby portions of cytoplasm. Unlike macroautophagy, which is acutely regulated by a lack of amino acids, microautophagy appears to account for nonselective basal proteolysis, i.e., is not regulated acutely (Ahlberg et al., 1985; Mortimore et al., 1988). In studies of long term starvation of rats, as basal protein degradation decreases the presence of type A lysosomes decreases while other microautophagic forms maintain their original total volume. This was taken to suggest that the type A lysosome serves to regulate basal autophagy (Mortimore

et al., 1988). The molecular machinery that causes the lysosomes to change shape to sequester and degrade portions of cytoplasm directly is completely unknown.

The morphological study of glucose adaptation in *P. pastoris* presented herein represents the first description of microautophagy in yeast. In fact, it appears that two types of microautophagy may be functional: selective autophagy induced by glucose in which the entire vacuole changes shape to engulf peroxisomes (Fig. 3-6, 6-1); nonselective autophagy of random cytoplasmic components induced by nitrogen starvation (see Fig. 5-6A,C, 6-2). The differences and similarities of these pathways have previously been discussed (see Table 5-1). My purpose here is to define hypothetical steps which may explain the phenomena involved in the two processes of microautophagy which are suggested by the morphological data.

P. pastoris cells which are in stationary phase contain clusters of peroxisomes and the vacuole is nearly spherical (6-1, "step 1"). Within one hour after the addition of glucose the vacuole is triggered to specifically recognize peroxisomes and starts to change shape to form a cup shape with the peroxisome clusters inside (6-1, "step 2"). Protein synthesis may be required for the change in vacuolar shape (see Fig. 5-2, 5-6D). The vacuolar cupping continues until the peroxisome cluster is completely surrounded by the vacuole (which has undergone fusion events with itself to become a hollow spheroid), but is still not within the lumen since the inner vacuole membrane is still intact (6-1, "step 3"). The final step to get the peroxisomes into the lumen of the vacuole is

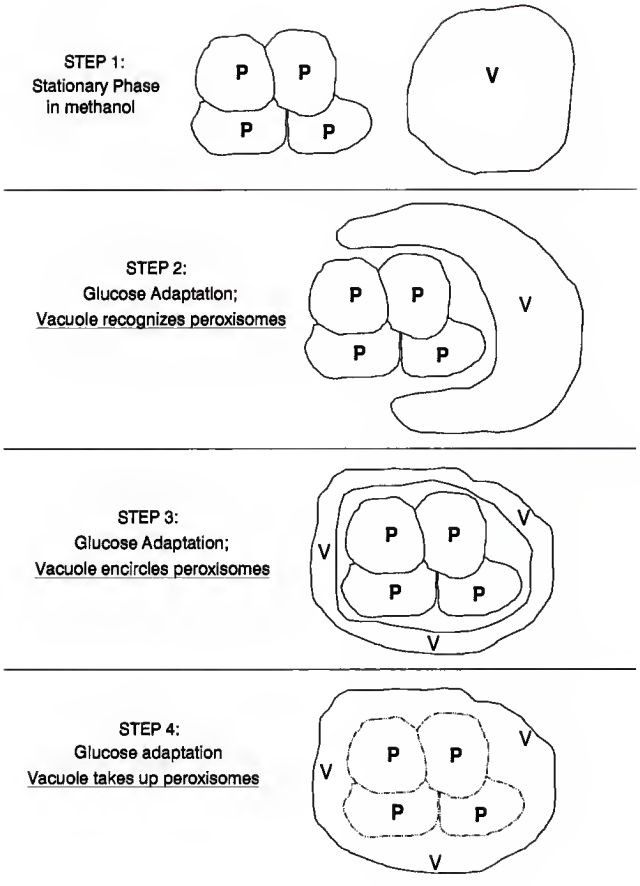

Figure 6-1. Proposed steps in microautophagic uptake of peroxisomes by the vacuole during glucose adaptation in *P. pastoris*. Upon addition of glucose to *P. pastoris* cultures at stationary phase in methanolic medium the vacuole progressively surrounds and engulfs clusters of peroxisomes and degrades them. See text for details. P = peroxisome; V = vacuole.

the disappearance of the inner membrane which makes the vacuolar lumen continuous with the sequestered components (6-1, "step 4"). This whole process occurs quite rapidly since all stages of the process are observed within the first hour of glucose adaptation. Further elucidation of the pathway awaits the three dimensional reconstruction of the process either by serial sectioning for transmission electron microscopy or development of real-time light microscopic methods to trace movements of the yeast vacuole.

Another possible form of microautophagy functions during nitrogen starvation in *P. pastoris*. During nitrogen deprivation, the limiting membrane of the vacuole changes shape on limited areas of its surface, forming invaginations or pits and finger-like flaps (6-2, "step 1"). These changes in the vacuolar membrane's shape require protein synthesis (see Fig. 5-6) as they do in the other form of microautophagy (see preceding paragraph). Cytoplasmic components (e.g., mitochondria, ribosomes, and soluble proteins) which are in the regions encompassed by these flaps and invaginations are sequestered into the vacuole as these pits close around them (see arrow in Fig. 5-6C), pinch off and become distinct bodies in vacuolar lumen (see Fig. 5-6C; 6-2, "step 2"). These autophagic bodies are apparently rapidly degraded since they accumulate in the vacuolar protease mutants but are only occasionally observed in the wild type cells during nitrogen starvation (compare Fig. 5-6A to 5-6C).

In summary, *P. pastoris* appears to be a model for two forms of microautophagy: selective microautophagy of large peroxisomes; and nonselective microautophagy of relatively small cytoplasmic components

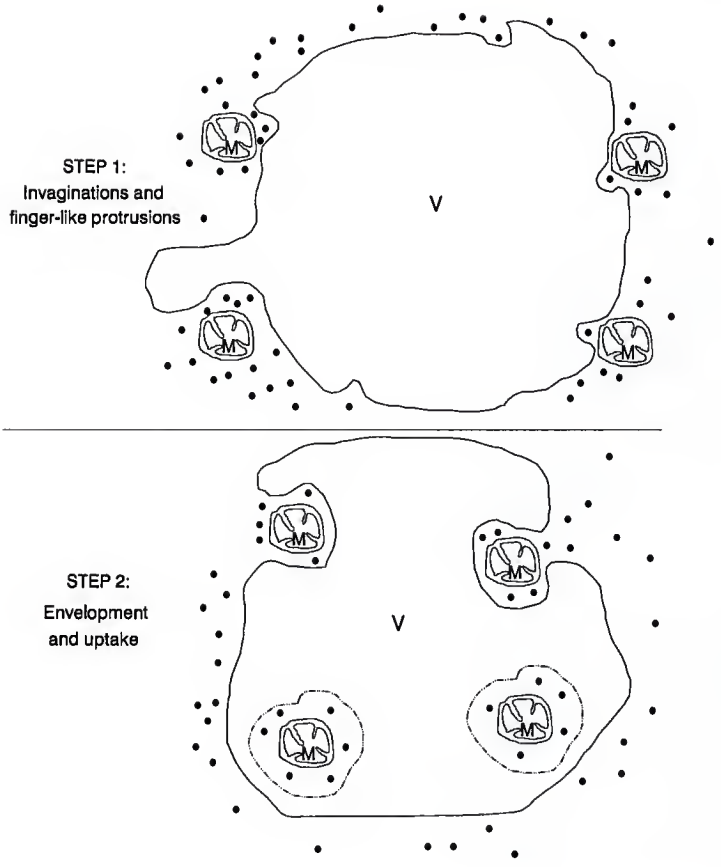

Figure 6-2. Proposed steps in microautophagic uptake of random cytoplasmic components during nitrogen deprivation in *P. pastoris*. Incubating *P. pastoris* cells in medium lacking a nitrogen source causes the vacuole to invaginate and extend finger-like protrusions. The cytoplasmic components which happen to occupy the areas surrounded by vacuolar extensions are taken up and degraded. See text for details. M = mitochondrion; V = vacuole; close circles represent free cytoplasmic ribosomes.

(mitochondria, ribosomes, and soluble proteins). These represent the first yeast models for microautophagy of which the latter, nonselective model produces microautophagic forms very similar to those seen in type A and type R lysosomes in mammals (Mortimore et al., 1988; see discussion of mammalian microautophagy above). Selective forms of microautophagy have not been shown to exist.

Macroautophagy

Depriving rat liver cells of amino acids or serum causes a large increase in cellular protein degradation and a corresponding increase in macroautophagic events (Mortimore and Pösö, 1987; Dunn, 1990a). In has been proposed that these acute regulators induce the nonselective degradation of cytoplasmic components. Portions of rough endoplasmic reticulum lose their associated ribosomes and invaginate to surround and envelop random portions of cytoplasm containing soluble proteins, peroxisomes, ribosomes and mitochondria to form autophagosomes (Dunn, 1990a). Subsequently, the autophagosomes acquire hydrolytic enzyme either directly from the Golgi or by fusion with primary lysosomes and thus become autolysosomes and degrade the sequestered components (Dunn 1990b).

A somewhat similar process has been shown to occur in *H. polymorpha* during glucose adaptation see Fig. 3-7; Veenhuis et al., 1983) and in *P. pastoris* during ethanol adaptation (Fig. 5-3). The apparent sequence of these events is depicted in schematic form with special reference to *P. pastoris* (Fig. 6-3). The

first morphologically distinguishable step is the sequestration of individual peroxisomes within a cluster into autophagosomes (Fig. 6-3, "step 1). The autophagosome is composed of a variable number of extra membrane layers closely surrounding and serving to sequester the peroxisome from the cytosol. The source of the autophagosomal membranes is unknown. Veenhuis et al. (1983) have suggested that they arise *de novo* in *H. polymorpha* since the pre-existing membranes were not observed in the process of wrapping peroxisomes; this would seem not to be the case in *P. pastoris* since autophagosome formation is independent of protein synthesis (see Fig. 5-2). Strands of membrane are often seen in the cytoplasm of *P. pastoris*, probably corresponding to endoplasmic reticulum or Golgi cisternae, and may serve to form the limiting membranes of autophagosomes.

The next step in macroautophagy in *P. pastoris* is the fusion of the autophagosome with the vacuole (6-3, "step 2). Presumably the autophagosomal membranes allow the recognition of and fusion with the vacuole. The outermost membrane layer fuses with the vacuolar membrane which causes the autophagosome lumen and vacuole lumen to be joined to allow ingress of the autophagosomal contents. This phenomenon is best seen in Figure 5-5d (see arrows, compare to arrows in Fig. 6-3, "step 2") in WDY2, which has a defect in microautophagy and so has to rely on the belated induction of macroautophagy.

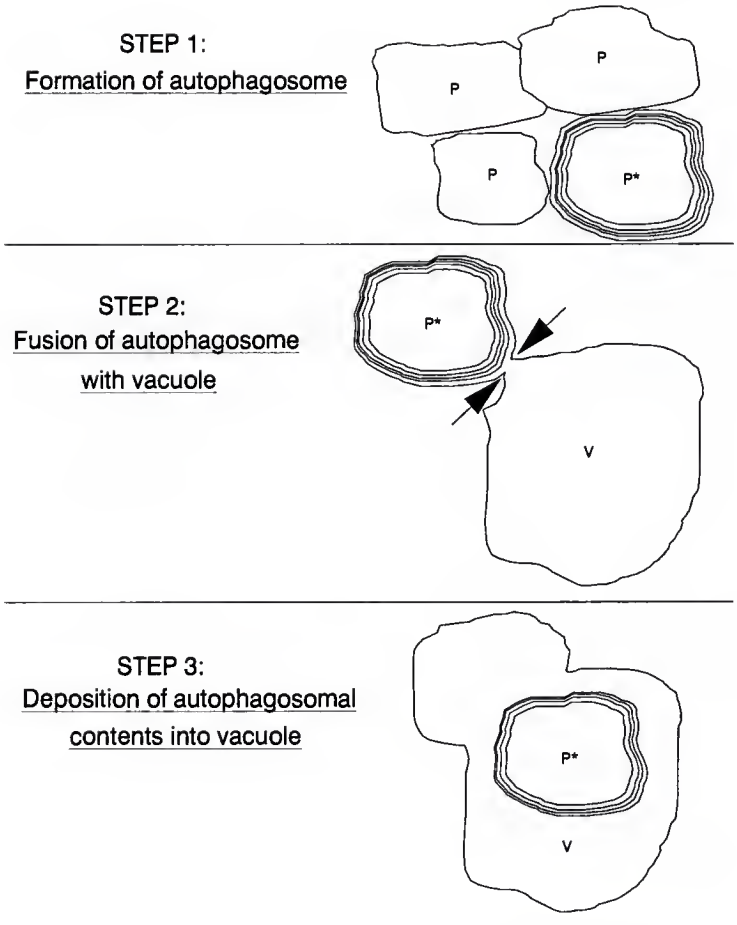

Figure 6-3. Proposed steps in macroautophagic uptake of peroxisomes by the vacuole during ethanol adaptation in *P. pastoris*. Upon addition of ethanol to *P. pastoris* at stationary phase in methanolic medium, individual peroxisomes are sequestered into autophagosomes which fuse with the vacuole where the sequestered peroxisomes are deposited for inactivation/degradation. See text for details. P = peroxisome; P* = peroxisome sequestered in autophagosome; V = vacuole; arrows point to region of fusion of autophagosome with vacuole.

The final morphologically identifiable step is the actual appearance of the residual autophagosome, containing a peroxisome and several layers of autophagosome membrane, in the vacuolar lumen (6-3, "step 3"). The retention of some of the autophagosomal membranes around the peroxisomes apparently protects the peroxisomal matrix proteins from degradation for several hours. This is suggested by the fact that AOX enzyme activity decreases rapidly during ethanol adaptation but AOX protein is degraded much more slowly (see Fig. 5-1). The loss in activity may be due to a relatively minor degradative event which removes the FAD prosthetic group from homo-octameric AOX (Bruinenberg et al., 1982; see discussion in Chapter 5). This minor degradative event may be possible within the residual autophagosome while total degradation of AOX protein may be impeded.

It is hoped that these working models of three forms of autophagy in *P. pastoris* will serve as a starting point for the further analysis of autophagy in yeast. These models should be further developed by three dimensional morphological analyses; by investigation of the origin of the autophagosomal membranes; and by discovering the proteins which subserve the processes of autophagic sequestration.

Divergent Pathways

The observed biochemical and ultrastructural processes of autophagy reported in the present paper suggest the existence of certain events occurring

at the molecular level which mediate the degradative pathways (depicted schematically in Figure 6-4). The various pathways undergo various modes of uptake into the vacuole, which is the ultimate location and site of degradation in all cases examined. The postulated phenomena in boxes probably each represent the actions of one or more unknown proteins or other molecules, the elucidation of which will lead to the understanding of the molecular mechanisms of autophagy.

Glucose Adaptation

Glucose or its catabolites are recognized by a signaling mechanism in stationary methanolic cultures of *P. pastoris*. This signal causes the synthesis of protein/proteins which must be present in order to initiate downstream events (cycloheximide prevents peroxisome and FDH degradation; see Fig. 5-2). The signal sets into motion a series of events which lead to the ultimate degradation of FDH and peroxisomes. Some change must occur in the vacuole so that it now recognizes FDH as a protein to be degraded. After recognition, FDH must be taken up into the vacuole for degradation since cells with proteolytically-deficient vacuoles do not degrade FDH (see Fig. 3-9). FDH may bind to peroxisomes and be taken up into the vacuole by microautophagy. Or FDH may be recognized by a heat shock protein, similar to prp73, which is synthesized only during glucose adaptation, which somehow facilitates entry of FDH into the vacuole, as is the case during serum deprivation in mammalian systems (Dice et al., 1990). Holzer and Purwin (1986) provided evidence that glucose initiates a cascade of events that leads to the phosphorylation of fructose-1,6-

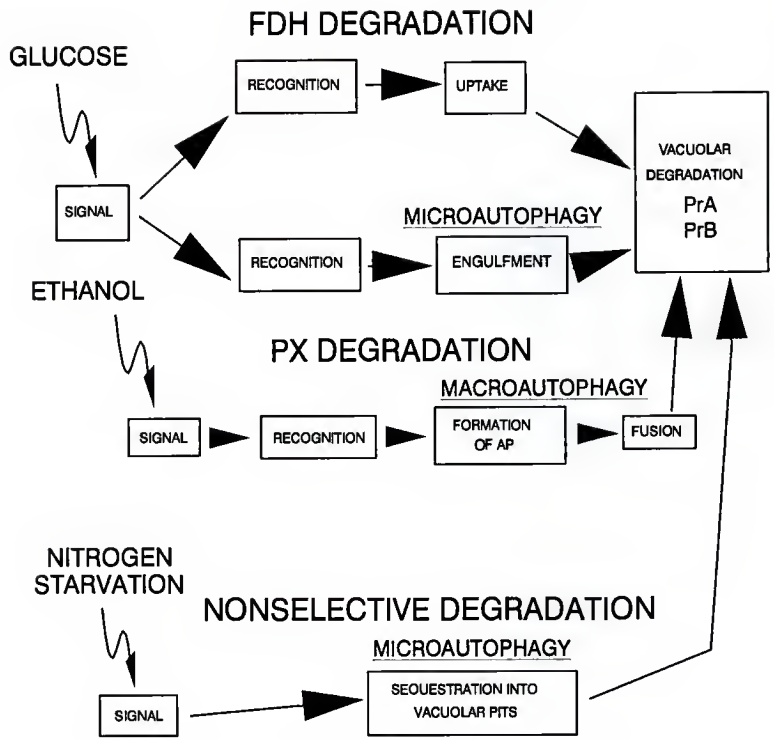

Figure 6-4. Postulated events mediated by unknown molecules which lead to the degradation of cytoplasmic components during glucose and ethanol adaptation and nitrogen starvation. The observed ultrastructural and biochemical phenomena suggest the occurrence of signaling, recognition, and visible events which are mediated by unknown molecules. These events are shown in boxes and represent the functions which are governed by these unknown molecules. See text for details. AP = autophagosome; PX = peroxisome; PrA = proteinase A; PrB = proteinase B.

bisphosphatase, targeting it for degradation. It is not known if similar events take place relative to FDH.

Similarly, changes must occur in the vacuole and/or peroxisome which allows the recognition of peroxisomes as destined for degradation. It appears that the peroxisomal membrane is necessary for recognition since, in peroxisomal mutants of *H. polymorpha* which form cytosolic AOX crystalloids lacking limiting membranes, AOX is not readily degraded in response to glucose (van der Klei et al., 1991). Proteins on the vacuolar membrane mediate recognition of the obsolete peroxisomes. Other proteins must respond to peroxisome recognition by causing the vacuole to change shape, cupping around peroxisome clusters and engulfing them. Degradation rapidly follows entry of peroxisomes into the vacuolar lumen.

Ethanol Adaptation

Ethanol also elicits a signal in methanol-induced *P. pastoris* which leads to the ultimate degradation of the AOX-containing peroxisomes (see Fig. 5-1). Proteins or second messengers signal the membranes which form the limiting membranes of autophagosomes to recognize peroxisomes. These membranes form autophagosomes by wrapping closely around individual peroxisomes by an unknown mechanism. These autophagosomal membranes probably contain protein(s) which allow the fusion of the autophagosome with the vacuole. An enzyme activated by PrA and/or PrB enters the residual autophagosomes in the vacuolar lumen and removes the covalently bound FAD from AOX, inactivating

it. Gradually other PrA/PrB-dependent enzymes degrade the entire autophagosome.

Nitrogen Starvation

P. pastoris cells cultured in media lacking a nitrogen source sense this starvation condition and the signaling mechanisms initiate the events which will lead to mating. One the first of these events is the initiation of vacuolar degradation. The starvation signal is communicated to the vacuole which responds by forming pits and flaps which envelop and sequester the cytoplasmic components which happen to be present. The proteins or other molecules which mediate the events of invagination, fusion to close off the pits and sequester cytoplasmic components, and budding from the vacuolar limiting membrane into the vacuolar lumen are unknown but all represent possible sites of mutation, and consequently, eventual elucidation. Again, PrA/PrB-dependent degradation of sequestered components follows rapidly.

In summary, I have postulated broad classes of events which most probably occur given the biochemical, morphological, and genetic evidence (see Fig. 6-4; Table 5-1). Different signals elicit different forms of autophagy; given that the forms of autophagy discussed are not constitutive, changes must occur which allow recognition of the peroxisomes or FDH by the sequestering elements during selective autophagy; the mode of sequestration differs in each case, suggesting different effector proteins; *gsa* mutants affect some but not all cases; some but not all cases are dependent on proteins synthesis; in all cases,

degradation/inactivation occurs in the vacuole. The boxes in Figure 6-4 delineate these broad events, each mediated by one or more proteins or other molecules. The boxed events represent possible sites for mutation, yielding autophagy-deficient mutants which can lead to the elucidation of the molecular mediators of the mechanisms of autophagy.

Prospects and Conclusions

P. pastoris is an important new model for several distinct modes of autophagy: selective microautophagy induced during glucose adaptation; selective macroautophagy induced during ethanol adaptation; and nonselective microautophagy induced during nitrogen starvation. The importance of this model derives from the limitations of the rat liver model which has been the predominant one to date. This model has been useful for the ultrastructural description of autophagy and for some aspects of its regulation, but none of the effector proteins which mediate autophagy in mammals has been identified. The model offered by *P. pastoris* should uncover the identity of effector proteins very rapidly through the use of autophagy-deficient mutants, leading to the cloning and sequencing of genes required for autophagy.

I have developed methods for the investigation of autophagy in *P. pastoris* which allow ultrastructural, biochemical, and genetic analysis of autophagy and precise control of the type of autophagy induced. Three dimensional morphological examination of normal cells will allow verification,

refutation, and refinement of the models of the ultrastructure of autophagy presented in Figures 6-1, 6-2, and 6-3. Most importantly, continued isolation of mutants which are defective in glucose- and ethanol- and nitrogen starvation-induced autophagy until the pathway is saturated, i.e., mutants have been isolated for all the proteins represented by the boxes in Figure 6-4, will lead to the identification of molecular mechanisms of autophagy. This type of analysis of *P. pastoris* is being conducted in the laboratories of S. Subramani and J.M. Cregg in regards to peroxisome biogenesis. Multiple complementation groups have been isolated and several genes cloned, including one which is functionally interchangeable with its human homolog (S. Subramani, personal communication).

I have isolated two complementation groups deficient in glucose-induced microautophagy which have already yielded information relevant to understanding microautophagy (see Chapter 5). Morphological evidence suggests that the defect in WDY2 is an inability of the vacuole to recognize peroxisomes or to change the shape in order to microautophagically sequester clusters of peroxisomes. Since this strain does respond to ethanol by slowly activating the macroautophagy pathway, this suggests that the ethanol signal is being received. Therefore, the normal GSA2 gene product probably functions downstream of the signal, in the recognition or sequestration step (see Fig. 6-4). The molecular bases of the recognition and sequestration steps is unknown at present. Further analysis of the gene products required for autophagy should

reveal the cellular machinery responsible for these phenomena. Possibly, the recognition of peroxisomes by the vacuole is mediated by specific guanine nucleotide-binding proteins. The ability of the vacuole to change shape to surround clusters of peroxisomes may be dependent on the actions of the cytoskeleton.

In addition, I have isolated genomic DNA which functionally complements the autophagy defect in WDY2 (see Appendix). Further analysis of this DNA may lead to the discovery of the identity and function of the first protein known to be required for the process of autophagy. Further isolation of *gsa* mutants followed by complementation analysis will eventually lead to the identification of many alleles of each complementation group with no new complementation groups appearing. This will be an indication that the pathway is saturated with mutations, i.e., all genes which are functional in the glucose-induced selective autophagy pathway will be present in mutant form in the various *gsa* strains. Biochemical, cell biological, and genetic analysis of an entire set of mutants which saturates the pathways of autophagy will lead to a complete understanding of the mechanisms of autophagy.

APPENDIX
ISOLATION OF GENOMIC DNA WHICH FUNCTIONALLY COMPLEMENTS THE AUTOPHAGY DEFECT IN WDY2

Introduction

In order to identify the gene affected in WDY2, several unique reagents or tools must be available: 1) a selectable marker in the yeast strain so that transformants can be detected; 2) a shuttle vector-borne genomic library suitable for functional complementation of the mutated gene; and 3) a screen for the rescued phenotype. The use of these tools along with the standard protocols which have been developed for DNA handling in yeast and *E. coli* makes possible the isolation of specific DNA segments with relative ease.

By transforming haploid mutants with a plasmid-borne genomic DNA library, then assaying for functional complementation (rescue) of the mutant phenotype, then recovering the DNA in rescued mutants, genes may be cloned and sequenced. This information leads to ready identification of the proteins active in the pathway of interest and further genetic manipulation and study of the proteins can yield data on protein localization, interactions with other cellular components, structure, and function. The specific aim of this part of the study was to functionally complement the glucose-induced selective autophagy (*gsa*) mutant strain WDY2 with a plasmid-borne genomic library and begin the

characterization of the DNA which is active in the process of selective autophagy.

Methods

Solutions and Media

Ampicillin Stock:

1. Make up in water at 50 mg/mL; filter sterilize.
2. Aliquot and store at -20°C.
3. Can re-use aliquots.

LB Broth (1 L):

5g yeast extract
10 g tryptone
5g NaCl
2.5 mL 1M KOH
1.5 g agar for plates
100 μg/mL ampicillin for LB/Amp (added from 50 mg/mL sterile stock when broth is cooled to ~50°C).

TE Buffer:

10 mM Tris/Cl (made to desired pH of final solution)
1 mM EDTA (pH 8.0 stock)

Phenol:

0.1% 8-Hydroxyquinoline was added to buffered phenol as an antioxidant.

Phenol/Chloroform/isoamyl alcohol:

Mix in the proportions 25:24:1, respectively using buffered phenol

Yeast DNA extraction buffer (Can be stored at room temperature for 1 year):

2% Triton X-100
100 mM NaCl
1 mM EDTA, pH 8.0
1% SDS
10 mM Tris/Cl, pH 8.0

YETM Medium, 500 mL:

2.5 g yeast extract
10 g tryptone
5 g $MgSO_4 \bullet 7H_2O$

pH to 7.5 w/ KOH
for plates: 7.5 g agar

TFB1, 200 mL:
0.59 g KOAc (30 mM)
2.42 g RbCl (100 mM)
0.29 g $CaCl_2 \bullet 2H_2O$ (10 mM)
1.98 g $MnCl_2 \bullet 4H_2O$ (50 mM)
30 mL glycerol (15%)
1. Adjust to pH 5.8 w/ 0.2 M acetic acid.
2. Bring to 200 mL with ddH_2O.
3. Sterilize by filtration.
4. Store at 4°C

TFB2, 200 mL:
0.42 g MOPS (10 mM)
2.21 g $CaCl_2 \bullet 2H_2O$ (74 mM)
0.24 g RbCl (10 mM)
30 mL glycerol (15%)
1. adjust to pH 6.5 w/ KOH
2. fill to 200 mL with ddH_2O
3. sterilize by filtration
4. store at 4°C

Procedures

Transformation of *E. coli* with plasmid DNA (RbCl method). Competent cells were prepared from fresh DH5α colonies streaked from frozen stock onto YETM plates and incubated overnight at 37°C. A single colony is plucked and used to inoculate a 5 mL YETM liquid culture which is incubated overnight at 37°C. One mL of this culture is used to inoculate 50 mL prewarmed YETM which is incubated with shaking for 2 h at 37°C. This culture is chilled for 5 min on ice and centrifuged for 10 min at 2000 x g at 4°C. The supernatant is immediately aspirated and the cells resuspended n 10 mL ice cold TFB1 with gentle trituration. The cells are kept for 5 min on ice then centrifuged at 2000 x

g for 10 min at 4°C after which the supernatant is immediately aspirated. The cells are resuspended in 2.0 mL ice cold TFB2 with gentle repipeting. The cells are kept on ice for 15 min, after which they are ready to be transformed or frozen in a dry ice/ethanol bath and stored at -80°C.

For transformation, the stored competent cells are thawed at room temperature then placed on ice for 10 min. 100 μL of competent cells are mixed with ~ 0.1 μg plasmid DNA in a volume of 10 μL TE. The mixture is incubated for 2 min on ice and then for exactly 90 sec at 42°C. The cells are returned to ice for 2 min after 1 mL of YETM is added and the cells are incubate at 37°C for 1 h with gentle shaking.

Transformation of *Pichia pastoris* with plasmid DNA (electroporation method). A single *P. pastoris* colony was grown in a 2 mL YPD preculture until saturated (~ 2d). A 500 mL culture of YPD in 2 L flask was inoculated with enough of preculture to get A_{600} = 0.6 to 1.0 after overnight culture. The cells were harvested at 4°C in sterile 250 mL centrifuge bottles at 4000 x g for 5min then resuspended in 100 mL YPD+ 2 mL 1 M HEPES, pH 8.0 to which 2.5 mL sterile 1 M DTT wasadded while swirling. The cells were incubated for 15 min at 30°C with gentle shaking. The cells were brought to 500 mL with sterile water and harvested as above then resuspended in 500 mL sterile cold water then harvested, then 250 mL sterile cold water and then harvested again. The cells were resuspended in 25 mL sterile cold 1 M sorbitol in a 50 mL sterile conical

tube at 2000 x g at 4°C for 10 min. Finally the cells were resuspended in 0.5 mL sterile cold 1 M sorbitol, a making a thick paste.

40 μL of these concentrated cells were mixed with 1-10 μg plasmid DNA in TE buffer in total volume of 5 μL in a 1.5 mL microfuge tube on ice. The mix was transferred to ice-cold 0.2 cm gap electroporation cuvette and tapped to the bottom of cuvette (Cuvettes: For BIO-RAD Gene Pulser; "Pulser Cuvette", 0.2 cm electrode gap. The cells were pulsed at 1.5 kV, 25 μF, 400Ω in Bio-Rad Gene Pulser (time constant was reported as ~8.0 msec). 1 mL ice-cold 1 M sorbitol was immediately added to the cuvette, gently mixed and transferred to a cold microfuge tube. Portions of the transformed yeast were spread directly on transformation plates and incubated at 30°C until colonies appeared (~3d).

Isolation of plasmid DNA from *P. pastoris* (for transformation of *E. coli*). Two mL of YPD or selective medium was inoculated with a single yeast colony and incubated until saturated. 1.5 mL of this culture was transferred to a microfuge tube and centrifuged 5 sec at room temperature; the supernatant was poured off and the cell pellet was disrupted pellet by vortexing briefly. The cells were resuspended in 200 μL yeast DNA extraction buffer + ~200 μL (0.3 g) 0.5 mm glass beads and 200 μL phenol/chloroform/isoamyl alcohol then vortexed for 2 min at highest speed. This mixture was microfuged at highest speed for 5 min at room temperature. The upper, aqueous layer was aspirated and 1 to 2 μL of this layer used to transform competent *E. coli* as above.

Results and Discussion

A *P. pastoris* genomic library was prepared by digesting total genomic DNA with Sau3IIA and the resulting DNA fragments were sized to 5 to 10 kb on cesium chloride gradients. The sized DNA was inserted into the unique BamHI site in the shuttle vector pYM8 (Fig. A-1; Cregg et al., 1985). The library was estimated to contain 50 replicates of the *P. pastoris* genome which should yield a 98% chance of containing all genes present in the genome (James M. Cregg, personal communication. The library was the very generous gift of J.M. Cregg, Oregon Graduate Institute).

The WDY2 strain was backcrossed 4 times and, accordingly, is a histidine auxotroph so that colonies which have been transformed with the library (which contains the HIS4 gene of *S. cerevisiae*) can be selected for on media lacking amino acids. The mutant strain was transformed by electroporation with 1 μg library DNA. The resulting transformants were screened by direct colony assay in the same manner as the *gsa* mutants were originally screened with one modification. Briefly, transformants were plated on MIM, incubated to allow colonies to form, then replica plated to glucose, incubated 14 h and screened for AOX activity. It was expected that only a few colonies would stay white, signifying the lack of AOX due to the restored ability to degrade peroxisomes. In fact, early attempts to identify rescued mutants yielded many white colonies, suggesting our assay was not sensitive enough. To increase the sensitivity, i.e., reliably identify all colonies which still retained AOX activity, the spheroplasting

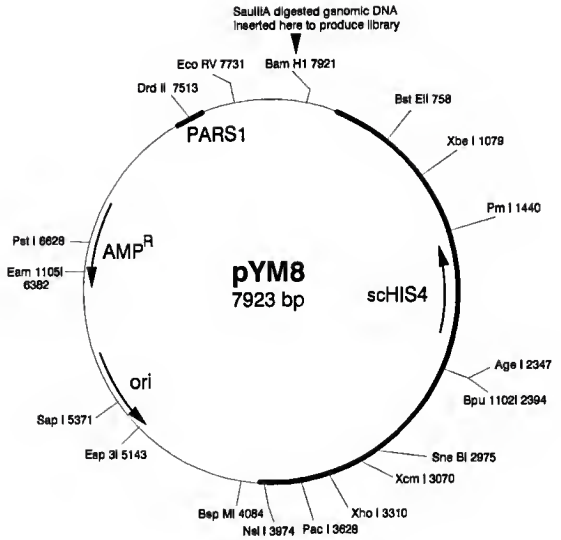

Figure A-1. Map of the *E. coli - P. pastoris* shuttle vector pYM8. pYM8 was constructed from the pBR322 *E. coli* plasmid (thin line) with the *S. cerevisiae* HIS4 gene inserted into the tetracycline resistance gene (scHIS4, thick line) and a 164 bp *P. pastoris* autonomous replication sequence inserted into the ClaI site of pBR322 (PARS1, thick line; Cregg et al., 1985). The BamHI site into which SauIIIA digested *P. pastoris* genomic DNA was inserted to produce a genomic library is indicated. AMP^R - ampicillin resistance gene; ori = *E. coli* origin of replication.

step of the colony assay was modified by utilizing twice as much Zymolyase 20T as in the original protocol (see Chapter 2).

Approximately 15,000 colonies were screened from which 14 positive colonies (i.e., lacking AOX activity) were selected. Verification of restoration of the ability to degrade peroxisomes was accomplished by the standard glucose adaptation protocol. Two of the 14 original, putatively rescued colonies exhibited wild type levels of peroxisome degradation after 6 h glucose treatment and were selected for analysis of their transforming library DNA. Plasmid DNA was extracted from these yeast strains (see Chapter2) and used to transform *E. coli*. Plasmid DNA was prepared from *E. coli* transformants using Wizard Mini-Preps (Promega) and subjected to restriction analysis using enzymes known to cut pYM8 once or not at all: BamHI, StuI, ApaI, BclI, BssHI, EcoRV, KpnI, MluI, PstI, and SpeI. The resulting DNA fragments were separated on 0.6% agarose gels and indicated that the plasmids carried by the two rescued WDY2 clones were identical and contained an insert of approximately 5.7 kb. The plasmid was designated pDLT1.

In order to provide evidence that this plasmid actually does contain the gene which is mutated in WDY2, the mutant strain was transformed with pDLT1 or pYM8 (as a negative control for complementation). Transformants were assayed for AOX activity during glucose adaptation in the usual way but the results were unexpected. In testing 6 pDLT1 transformants from two separate transformations, the ability of the cells to degrade peroxisomes was quite

variable. Some clones appeared to degrade peroxisome better than wild type while others were not as efficient. In addition, pYM8 itself appeared to partially rescue the autophagy-deficient phenotype. It was hypothesized that the reason for the inconsistent results with pDLT1 may be that some of the transformants carried integrated copies of the plasmid while some remained autonomous. The result of this could be that in the cultures carrying autonomous pDLT1, a subset of the cells were randomly losing the plasmid and thereby becoming histidine auxotrophs again. Since the cells were in media lacking histidine, nonselective (starvation-induced) autophagy would be turned on, perhaps causing nonselective turnover of peroxisomes in addition to selective peroxisome turnover and resulting in greater than normal peroxisome degradation (see Fig. 3-11; Fig. 4-3). Variation in the number of cells losing or retaining pDLT1 could cause the observed variation in results.

To test this hypothesis, transformants in which pDLT1 had become integrated in the genome were selected for in the following way. First, the transformants were grown on nonselective medium (YPD) overnight to encourage the loss of autonomous plasmid. A small sample of this culture was inoculated back into selective medium (lacking amino acids) and incubated overnight, then plated onto nonselective agar plates. After colonies appeared, they were replica plated onto selective medium and incubated until colonies formed. It was assumed that, if all the colonies appeared on both selective and nonselective media, then the transformants must have the plasmid stably

integrated into the genome (J.M. Cregg, personal communication). This procedure was successful and colonies were selected from this pool of stable transformants for further glucose adaptation assays.

The other problem which has been noted was that pYM8 conferred a partially rescued phenotype. This plasmid contains only 164 bp of *P. pastoris* DNA so was not expected to integrate into the genome by homologous recombination. The partially rescued phenotype could therefore be due to starvation-induced nonselective degradation in transformants which had lost the plasmid, as discussed above. It was decided that a more appropriate control would be cloned transformants containing integrated copies of plasmids with random library inserts. The strains which were produced by integrating pDLT1 or a random genomic insert-containing plasmids were analyzed for the ability to degrade methanol-induced peroxisomes in response to glucose (Fig. A-2). Three strains containing integrated copies of pDLT1 were able to degrade peroxisomal AOX at the wild type rate (Fig. A-2, pDLT1-1,2,3); strains containing integrated copies of random plasmids from the library were still deficient in the ability to degrade AOX (Ctl-1,2). These results suggest that, when integrated, pDLT1 has the ability to complement the autophagy defect in WDY2, while random library plasmids do not. Further restriction analysis of pDLT1 suggests the restriction map of the genomic library insert depicted in Fig. A-3.

In conclusion, a plasmid carrying a 5.7 kb fragment of *P. pastoris* genomic DNA was shown to functionally complement the *gsa* defect in WDY2 when

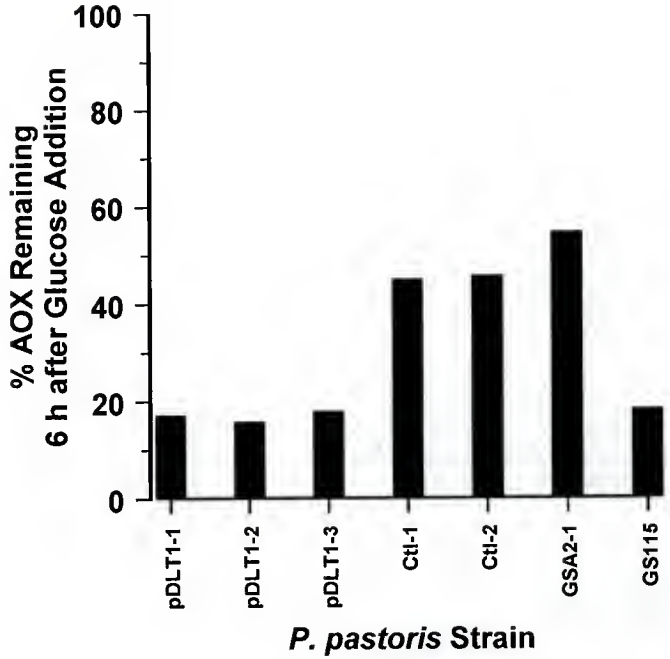

Figure A-2. Test of the ability of integrated pDLT1 to complement the autophagy defect in WDY2. WDY2 was transformed with pDLT1 or random library plasmids (Ctl-1,2) and clones containing integrated copies of the plasmids were selected (see text for details). Glucose adaptation assays were performed on 3 different clones carrying integrated copies of pDLT1 (pDLT1-1,2,3). Controls included 2 different clones carrying integrated copies of random library plasmids (Ctl-1,2), the mutant strain WDY2, and wild type GS115.

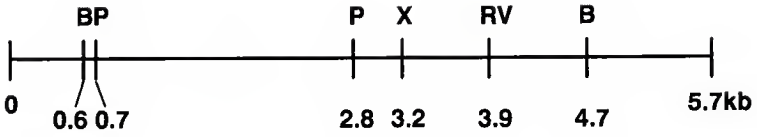

Figure A-3. Proposed restriction map of genomic library insert in pDLT1. Restriction enzyme digests were conducted on the 5.7 kb insert in pDLT1 suggesting the proposed map shown. Numbers below the line represent distance from the 5' end of the insert in kilobases (kb). B = BamHI; P = Pst1; X = XhoI; RV = EcoRV.

integrated into the genome (see Fig. A-2), suggesting that it contains a wild type copy of the gene which has been mutated in WDY2.

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BIOGRAPHICAL SKETCH

I was born in Hammond, Indiana, on November 20, 1955. My mother, Mary Arden Jones Tuttle, was a native of Texas and my father, Wesley Earl Tuttle, was from Kentucky. I was a varsity wrestler for three years at Griffith High School (Griffith, Indiana) and graduated from there in 1973. I then worked on my parent's farm in Ohio County, Kentucky, and in industry in Indiana for the following nine years before beginning college at Oklahoma State University in 1982. I stayed there for one year then transferred to the University of Kentucky from which school I graduated with the degree Bachelor of Science in Agriculture with high distinction in 1986. I next attended Texas A&M University and earned a Master of Science degree in reproductive physiology in 1989. In May 1990 I entered the Department of Anatomy and Cell Biology in the College of Medicine at the University of Florida and defended my dissertation in December 1994.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May 1995

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